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TI ***Minimization*** of shaking-induced formation of insoluble ***aggregates*** of ***insulin*** by cyclodextrins
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Minimization of Shaking-induced Formation of Insoluble Aggregates of Insulin by Cyclodextrins

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Aggregation is known to complicate insulin delivery and the processing and formulation of biotechnology-derived peptide/protein drugs. Shaking-induced formation of insoluble aggregates in bovine insulin and the potential role of cyclodextrins in preventing such aggregation were investigated. Insulin, dissolved in phosphate buffer, pH 7.2, and preserved with 2 mg/ml of phenol was aggregated, in triplicate, by shaking at 450 rpm for 2.5 days on a gyrotory shaker. Visible aggregation was quantitated by measuring optical density in the visible range on a spectrophotometer. Solutions were then filtered through a 0.22 μ filter and the amount of insulin remaining in filtrate was determined by HPLC. Aggregation increased at lower concentrations, with solutions turning milky at 0.5 mg/ml; HPLC assay of filtrate indicated a complete loss of insulin. Under the same conditions, except for shaking, control solutions exhibited no insulin loss, excluding absorption as a cause of the insulin loss. The use of cyclodextrins (0.5 mg/ml) to stabilize insulin was investigated. α-, β-, γ- and hydroxypropyl-β-cyclodextrin, each at 1.5% level, were used to prevent aggregation. The efficacy of cyclodextrins in preventing aggregation (% insulin aggregated in parentheses), was: hydroxypropyl-β- (15) ~ β- (18) > α- (54). No protection was observed with γ-cyclodextrin.

KEYWORDS: aggregation, cyclodextrins, insulin

INTRODUCTION

Artificial delivery systems and implantable devices are now being used in humans receiving long-term insulin treatment. A major problem associated with these techniques is the blockage of tubings, membranes and pumps following the aggregation of insulin into precipitates (Lougheed et al.,1980). The nature of these insoluble aggregates is described by Brange (1987). The systems require frequent flushing, which may not be possible with implanted systems. Aggregation may also lead to loss of biological potency. The objective of the present work was to characterize the shaking-induced formation of insoluble aggregates of insulin and select experimental conditions conducive to aggregation to enable accelerated screening of the potential usefulness of cyclodextrins in preventing or minimizing aggregation. Bovine insulin (zinc content 0.5%) was used and the present results, therefore, may not be extrapolated to human recombinant insulin without further studies. The choice of buffers and other experimental conditions will also have affected the aggregation potential. Insulin has a marked tendency to undergo self-association into dimers, hexamers or other soluble oligomers (Bi et al., 1984). Such soluble aggregates were not the subject of the present investigation, which was concerned only with insoluble aggregates.

The insoluble aggregates, or precipitates, of insulin formed in infusion pumps or similar devices may be studied in the laboratory by shaking insulin solutions to induce aggregation. This technique creates aggregates in a dispersed form, enabling them to be observed by the turbidity or milkiness of the solution. Attempts to prevent such aggregation by the use of additives are not new and investigators have used amino acids (Bringer et al., 1981; Quinn and Andrade, 1983), urea (Sato et al., 1983), serum (Albisser et al., 1980) and surfactants (Lougheed et al., 1983). Some studies of insulin soluble aggregates include the use of EDTA or bile salts to produce dissociation of hexamers to dimers or dimers to monomers (Liu et al., 1991; Li et al., 1992). The preparation of a non-aggregating sulfated insulin has also been reported (Pongor et al., 1983).

Cyclodextrins have recently been investigated

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to stabilize peptide/protein drugs (Brewster et al., 1991). The natural cyclodextrins, α-, β- and γ-, are cyclic oligosaccharides of 6, 7 and 8 glucopyranose units, respectively. The ring structure resembles a truncated core and the basis of their pharmaceutical use is their ability, owing to the hydrophobic property of the cavity, to form inclusion complexes. Current research has indicated that cyclodextrin derivatives given parenterally exhibit higher solubility and lower toxicity (Szejtli, 1991) while preserving their molecular encapsulation capacity. Hydroxypropyl-β-cyclodextrin, with a solubility > 50%, is especially promising as a parenteral excipient. The ability of α-, β-, γ- and hydroxypropyl-β-cyclodextrin to prevent or minimize the shaking-induced aggregation of insulin was investigated.

MATERIALS AND METHODS

Bovine insulin (zinc content about 0.5%) was procured from Sigma Chemical Company (St. Louis, MO). The α-, β-, γ- and hydroxypropyl-β-cyclodextrins were a gift from Wacker Chemicals (USA) Inc. All other chemicals used were of reagent grade. Bovine insulin was dissolved in phosphate buffer, pH 7.2, with and without cyclodextrins and preserved with 2 mg/ml of phenol. Three identical 25 ml conical flasks, each containing 4 ml of solution, were then shaken at 450 rpm in a Gyrotory® shaker (New Brunswick Scientific Co., NJ) to generate excessive air-water interfaces. Shaking was continued under ambient conditions for 2.5 days. At the end of this period, aggregation was visible as turbidity/milkiness, which was quantitated at 340 nm on a spectrophotometer (Beckman); increasing aggregation resulted in a higher optical density. Solutions were then filtered through a 0.22 μ filter in a stainless steel housing attached to a glass syringe, removing all insoluble aggregates. The clear filtrate was analysed by HPLC to quantitate the insulin that had not aggregated.

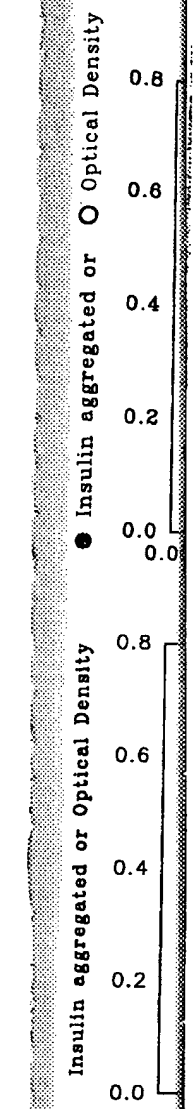
HPLC assay for insulin was performed using a solvent delivery system (Micromeritics, Model 750) and an Autosampler (Micromeritics 728) attached to a variable wavelength UV detector (Micromeritics 788) and an integrator (Hewlett Packard). Separations were performed on a μ Bondapak® C_{18} column with a pore size of 125 Å, using a mobile phase consisting of buffer: methanol:acetonitrile (40:50:10) at a flow rate of 2.0 ml/min; the detection wavelength was 215 nm. The buffer consisted of 0.05 M sodium sulfate and 0.05 M $NaH_2PO_4.H_2O$, adjusted to pH 3.2 with phosphoric acid. Since soluble aggregates of insulin were not of interest, techniques such as size exclusion chromatography or circular dichroism were not used.

RESULTS AND DISCUSSION

Air/water interfaces, such as those generated by shaking, provide a hydrophobic interface, causing adsorption followed by complete or partial unfolding of the protein molecule, thus exposing inner hydrophobic regions. The protein then aggregates through the interactions of the exposed hydrophobic regions. This mechanism was used to explain the results of the present investigation as follows:

Aggregation and Insulin Concentration

Optimum experimental conditions for accelerated aggregation were created as already outlined. A milky solution indicating substantial aggregation resulted at concentrations ≤ 0.5 mg/ml, as seen by increased optical density at 340 nm. The filtrate obtained did not give an HPLC peak for insulin, indicating complete loss of insulin by aggregation. A triplicate set of control solutions, concentration 0.5 mg/ml, was used under identical conditions (including filtration), except that they were not shaken. The controls exhibited no insulin loss, thereby excluding adsorption to glass or other factors as the cause of insulin loss under the conditions applied. At higher insulin concentrations aggregation demonstrated by the optical density data was reduced (Figure 1). A possible explanation could be the surfactant effect of the protein itself: as the surface becomes saturated at higher concentrations, a diminishing quantity of the protein undergoes surface interactions, thus decreasing the percentage of protein aggregated. However, the amount of insulin aggregated, determined by subtraction of the amount of insulin in the filtrate (measured by HPLC) from the total volume, did not follow the same profile (Figure 1). The explanation of this discrepancy may be that light scattering is dependent not only on the concentration, but also on the size of the



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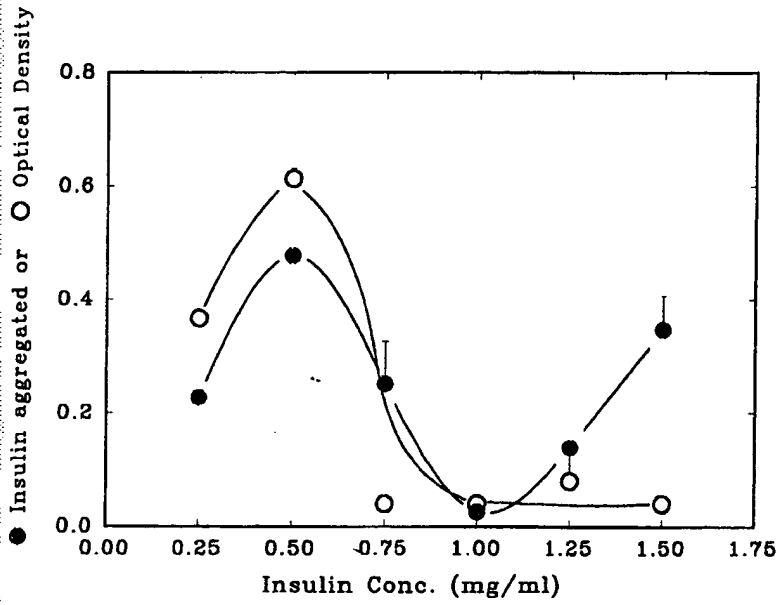

FIGURE 1. Effect of increasing concentration of insulin on shaking-induced aggregation as monitored by optical density at 340 nm or by the amount of insulin aggregated (mg).

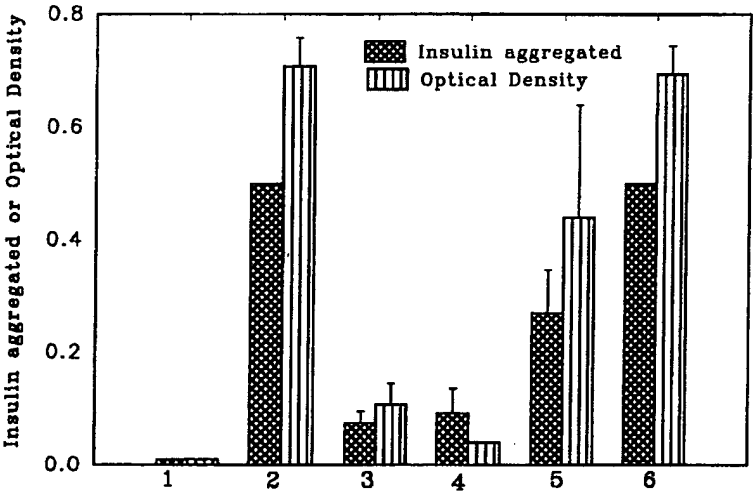

Figure 2

1: Control (no shaking)
2: Positive Control (shaking in absence of cyclodextrin)
3: Hydroxypropyl-beta-cyclodextrin
4: Beta-Cyclodextrin
5: Alfa-Cyclodextrin
6: Gamma-Cyclodextrin

FIGURE 2. Effect of α-, β-, γ- and hydroxypropyl-β-cyclodextrin on the shaking-induced aggregation of insulin (0.5 mg/ml) as monitored by optical density at 340 nm or by the amount of insulin aggregated (mg). A control with no shaking and a control with shaking in absence of cyclodextrins is included.

particles. At higher concentrations, size distribution, rather than the surface effect, may be sufficiently different to result in lower optical density.

Effect of Cyclodextrins on Insulin Aggregation

Owing to the solubility limits of β-cyclodextrin (max 1.85%), all the cyclodextrins were evaluated at a concentration of 1.5%. As seen in Figure 2, all cyclodextrins (except γ-) were able to minimize aggregation, with β- and hydroxypropyl-β-cyclodextrin being most effective. Both HPLC and optical density data indicate that cyclodextrins reduced aggregation. The efficacy of cyclodextrins in preventing aggregation (% insulin aggregated

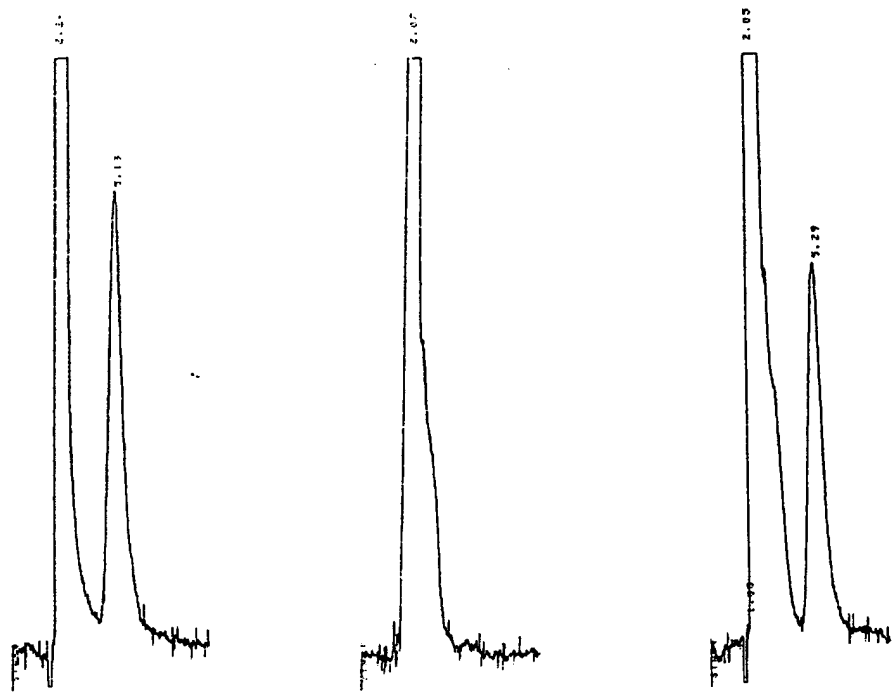

FIGURE 3. HPLC profile of an unaggregated sample of insulin (0.5 mg/ml) and of a sample following shaking in the presence and absence of hydroxypropyl-β-cyclodextrin.

in parentheses), was: hydroxypropyl-β- (15) ~ β- (18) > α- (54). No protection was observed with γ-cyclodextrin. Insulin that was shaken while being protected by hydroxypropyl-β-cyclodextrin exhibited about 85% retention of insulin as indicated by the HPLC peak compared with that obtained for the unaggregated sample. Conversely, the unprotected solution lost its entire insulin peak upon aggregation (Figure 3).

As suggested earlier, aggregation results when a protein is adsorbed and then unfolds at the air/water interfaces generated by shaking, exposing the hydrophobic amino acids that are normally buried in the interior. The exposed hydrophobic amino acid side chains of one molecule interact with those of another to form aggregates. The mechanism by which cyclodextrins minimize insulin aggregation is likely to be molecular encapsulation of these side chains, preventing these hydrophobic interactions. Aromatic amino acids are known to form a inclusion complex with cyclodextrins (Tokumura et al., 1986; Matsuyama et al., 1987). Several cyclodextrin molecules are likely to interact with one insulin molecule at the sites where aromatic amino acids (phenylalanine and tyrosine, in the case of insulin) reside, e.g. Phe (B25), which occurs at the subunit interface. Simpkins (1991) has reported that 20 to 40 molecules of hydroxypropyl-β-cyclodextrin interact with one molecule of interleukin-2 (IL-2), a protein with 133 amino acids. Although complexation did not affect the bioactivity of IL-2 in this study, the effect of cyclodextrins on the biological activity of insulin is not known.

The varying effectiveness of cyclodextrins in minimizing insulin aggregation may be related to their cavity diameter or solubility. The cavity diameters of α-, β- and γ-cyclodextrin are 5, 6 and 8 Å, respectively. The cavity diameter of γ-cyclodextrin is perhaps too large effectively to interact with the side chains of aromatic amino acids, which may explain its failure to prevent

insulin aggregation. Although more than one cyclodextrin may be acceptable in minimizing aggregation, the preferred agent for such use is hydroxypropyl-β-cyclodextrin, since it is the most soluble and least toxic derivative.

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TI Differential effects of cyclodextrin derivatives on aggregation and thermal behavior of ***insulin***
AU Tokihiro, Keiichi; Irie, Tetsumi; Uekama, Kaneto

SO Proceedings of the International Symposium on Cyclodextrins, 8th, Budapest, Mar. 31-Apr. 2, 1996 (1996), 357-360. Editor(s): Szejtli, J.; Szente, L. Publisher: Kluwer, Dordrecht, Neth.

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DIFFERENTIAL EFFECTS OF CYCLODEXTRIN DERIVATIVES ON AGGREGATION AND THERMAL BEHAVIOR OF INSULIN

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ABSTRACT

Maltosyl-β-cyclodextrin (G2-β-CyD) suppressed the aggregation of insulin in a neutral solution, while the sulfate of α-CyD (S-α-CyD) accelerated the aggregation. On the other hand, the sulfobutyl ether of β-CyD (SBE-β-CyD) showed differential effects on the insulin aggregation, depending on the degree of substitution; *i.e.* the inhibition at relatively low substitution and acceleration at higher substitution. Differential scanning calorimetric (DSC) studies indicate that the self-association of insulin stabilized the native conformation of the peptide, as indicated by an increase in the mean unfolding temperature (Tm). G2-β-CyD and SBE-β-CyD decreased the Tm value of insulin oligomers, while S-α-CyD increased the Tm value. These results suggest that a proper use of the CyD derivatives is effective in designing rapid and long-acting insulin preparations.

1. INTRODUCTION

Our previous studies have shown that hydrophilic CyDs including G2-β-CyD and 2-hydroxypropyl-β-CyD significantly inhibited the adsorption of bovine insulin to hydrophobic surfaces of containers and its aggregation by interacting with hydrophobic amino acid residues of the peptide [1]. Recently, the sulfates and sulfoalkyl ethers of CyDs have been evaluated as a new class of parenteral drug carriers, because they are highly hydrophilic and less hemolytic than the parent CyDs [2]. In the present study, we examined the effects of the CyD derivatives on the aggregation and thermal behavior of insulin in both acidic and neutral solutions by means of the ultrafiltration method, DSC and liquid chromatography-mass (LC/MS) spectrometry.

2. MATERIALS AND METHODS

2.1. Materials

Bovine insulin (27.5 IU/mg) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). G2-β-CyD, S-α-CyD with an average degree of substitution of 11.4 and SBE-β-

J. Szejtli and L. Szente (eds.), Proceedings of the Eighth International Symposium on Cyclodextrins, 357–360.

CyD with average degrees of substitution of 4.0 and 7.0 (SBE4-β- and SBE7-β-CyD: were donated by Ensuiko Sugar Refining Co. Ltd. (Yokohama, Japan), Kokusa Chemical Co. Ltd. (Tokyo, Japan), and CyDex L.C. (Overland Park, KS, USA respectively.

2.2. Methods

The aggregation of insulin was evaluated by measuring the remaining insulin in th filtrate (filter: DISMIC-13CP045AN; Advantec Co., Tokyo, Japan) after standin freshly prepared insulin solutions (0.15 mM, in pH 6.8 phosphate buffer) in a siliconc coated glass tube at 25°C. The DSC thermograms of insulin solutions were recorded b a MC-2 microcalorimeter (MicroCal, Inc., Amherst, MA, USA). The LC/MS spectra o insulin solutions were measured by a M-1200H LC/MS system (Hitachi, Tokyo, Japan equipped with an electrospray ionization (ESI) source.

3. RESULTS AND DISCUSSION

3.1. Effects of CyD Derivatives on Aggregation of Insulin

Insulin in a neutral solution (pH 6.8) was mostly assembled as zinc-containing hexamers eventually leading to the precipitation of higher order aggregates of the peptide in a concentration- and time-dependent manners. As shown in Fig. 1, G2-β-CyD significantly suppressed the aggregation of insulin, while S-α-CyD accelerated the insulin aggregation. G2-β-CyD may interact with hydrophobic amino acid residues of insulin, and thus prevent the aggregation by eliminating intermolecular hydrophobic contacts [1]. Since S-α-CyD has highly concentrated negative charges located near the entrance of the cavity and shows limited inclusion ability, the neutralization of cationic charges in insulin by S-α-CyD may contribute to the accelerated aggregation of the peptide.

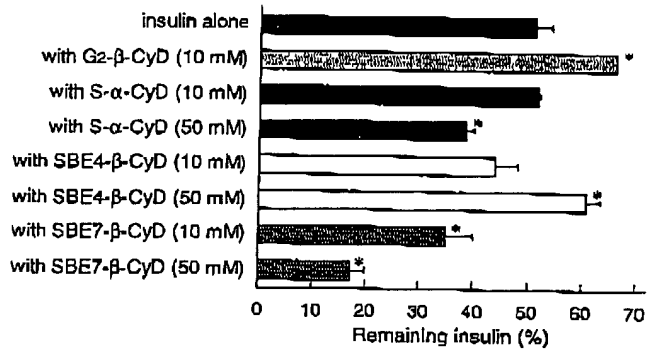

Fig. 1 Effects of CyD Derivatives on Aggregation of Insulin 24 h after Preparation of Insulin Solution (0.15 mM) in Phosphate Buffer (pH 6.8) at 25°C

Each point represents the mean±S.E. of 2-14 experiments.
*$p<0.05$ *versus* insulin alone.

On the other hand, SBE-β-CyD showed differential effects on insulin aggregation, depending on the degree of substitution; *i.e.* inhibition at relatively low substitution and acceleration at higher substitution. Since the sulfonate groups in SBE-β-CyD are appropriately spaced from the CyD cavity with an butyl chain and do not interfere with the inclusion process, SBE4-β-CyD may inhibit the insulin aggregation in a manner similar to G2-β-CyD. In case of SBE7-β-CyD, the electric effects seem to be more of a factor than the inclusion effects, eventually leading to the acceleration of the insulin aggregation. The differential effects of the CyD derivatives on the insulin aggregation were confirmed by the ultrafiltration experiments, in which G2-β-CyD and SBE4-β-CyD facilitated the permeation of insulin through the ultrafiltration membranes, while S-α-CyD and SBE7-β-CyD reduced the membrane permeation of insulin. Furthermore, G2-β-CyD facilitated the permeation of insulin through the membranes in an acidic solution (pH 2.0), in which insulin existed mainly as a zinc-free dimer in this condition, indicating that G2-β-CyD affects the equilibrium between the dimer and the monomer.

3.2. Effects of CyD Derivatives on Thermal Behavior of Insulin

The DSC thermograms of insulin solutions showed that self-association of insulin stabilized the native conformation of the peptide, as indicated by an increase in Tm. Fig. 2 shows the effects of the CyD derivatives on DSC thermograms of insulin in phosphate buffer (pH 6.8). G2-β-CyD and SBE-β-CyD significantly reduced the Tm value of insulin oligomers, the former being more effective. These CyD derivatives may shift the equilibrium in favor of the unfolded insulin by dissociating the oligomers and/or binding to hydrophobic side chains exposed on the unfolded peptide. On the other hand, S-α-CyD increased the Tm value of insulin, reflecting on the higher degree of association of the peptide.

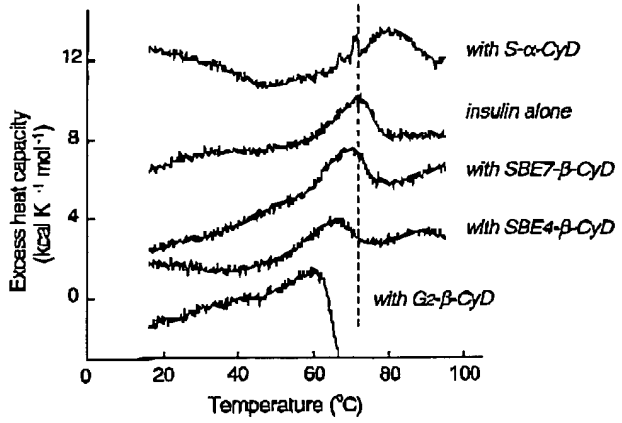

Fig. 2 Effects of CyD Derivatives (0.1 M) on DSC Thermogram of Insulin (0.1 mM) in Phosphate Buffer (pH 6.8)

The complexation of insulin with G2-β-CyD was further confirmed by the LC/M analysis. Fig. 3 shows the positive ion ESI mass spectra of insulin in the absence an presence of G2-β-CyD in acidified mixtures of water and methanol. In the absence c G2-β-CyD, insulin gave a bell-shaped multiple charge state distribution to the $(M+6H)^{6}$ multiple-protonated species. In the presence of G2-β-CyD, a peak corresponding to th complex or electrostatic adduct of the charged insulin with G2-β-CyD at a molar ratio c 1:1 was observed.

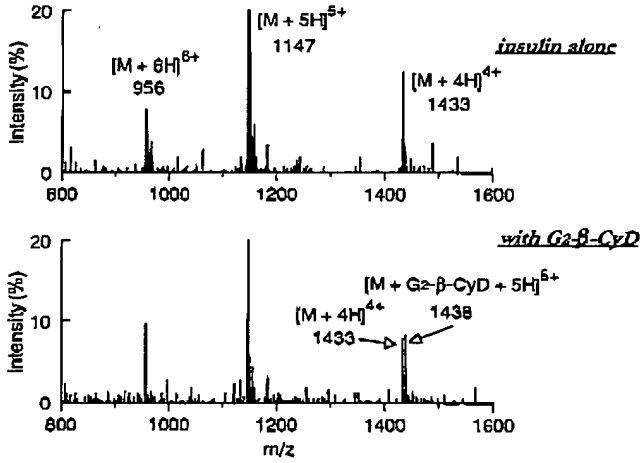

Fig. 3 ESI Mass Spectra of Insulin (0.1 mM) in Positive Ion Mode in the Absence and Presence of G2-β-CyD (2 mM) in Water/Methanol/Acetic Acid (47 / 47 / 6) Solution

4 CONCLUSION

The present results indicate that the CyD derivatives interact with insulin in a differential manner and hence a proper use of the CyD derivatives is effective in designing rapid and long-acting insulin preparations.

ACKNOWLEDGEMENTS

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TI Physical stability of ***insulin*** formulations
AU Lougheed, W. D.; Albisser, A. M.; Martindale, H. M.; Chow, J. C.; Clement, J. R.

SO Diabetes (1983), 32(5, Pt. 1), 424-32

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Physical Stability of Insulin Formulations

W. D. LOUGHEED, A. M. ALBISSER, H. M. MARTINDALE, J. C. CHOW, AND J. R. CLEMENT

SUMMARY

Insulin aggregation remains a fundamental obstacle to the long-term application of many insulin infusion systems. We here report the effects of physiologic and nonphysiologic compounds on the aggregation behavior of crystalline zinc insulin (CZI) solutions. Under conditions chosen to simulate the most severe that would be encountered in delivery systems (presence of air, continuous motion, and elevated temperature), both highly purified and regular CZI at 5 U/ml formed turbid gels in 5 days. At concentrations of 100 and 500 U/ml stability was increased with turbid gels forming at 12 and 15 days, respectively. Under identical conditions, 5 U/ml CZI formulations containing the physiologic surfactant lysophosphatidylcholine (0.02%) or the synthetic surfactants SDS (1%), Brij 35 (0.1%), Tween (0.01%), or Triton X (0.01%) retained a transmittance at 540 nm of >96% for 67–150 days. These nonionic and ionic surfactants containing the hydrophobic group, $CH_3(CH_2)_N$, with N = 7–16, remarkably stabilized CZI formulations while those lacking such groups demonstrated little or no effect. The alcohols glycerol (30–50%) and isopropanol (10–50%) were moderately effective stabilizers. Silicone rubber drastically accelerated aggregation in all but one formulation (1% SDS). Emphasis in this study was placed on the properties of 5-U/ml formulations. Controls run at higher concentrations indicated a positive correlation between concentration and stability. It was concluded that the aggregation of insulin into high-molecular-weight polymers may be inhibited by reducing the effective polarity of the solvent. In this regard, anionic and nonionic surfactants containing appropriately long hydrophobic groups demonstrated the greatest degree of stabilization. Finally, of all the medical grade materials likely to be used in pumps, silicone rubber is the most active in promoting insulin aggregation. DIABETES 32:424–432, May 1983.

From the Hospital for Sick Children (W.D.L. and A.M.A.), Toronto, Ontario, Canada, and Connaught Laboratories Limited (J.C.C., J.R.C., and H.M.M.), Willowdale, Ontario, Canada

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Many "open-loop" systems have been designed for the continuous infusion of insulin to diabetics. These permit predetermined doses of the hormone to be delivered by a variety of routes[1] (intravenous, intraperitoneal, and subcutaneous) with the ultimate goal of alleviating the complications of diabetes through improved glycemic control. The designs of these devices and their potential advantages have been reviewed in the literature.[2–9]

Unfortunately, the tendency of insulin to aggregate during storage in and delivery from these devices remains one of the fundamental obstacles to their prolonged clinical use.[10] With subcutaneous infusions in which the insulin reservoir is changed every 1–2 days, this has not been a major problem. However, in systems where replacement is less frequent, the aggregation of insulin into highly insoluble amorphous/crystalline polymers results in the blockage of tubings, membranes, and pumps.

In studies with one such device, Irsigler[11] observed that insulin aggregation was the inevitable end result when the peptide was placed in continuous motion and occurred irrespective of the materials employed in the pumping system. However, it would appear that small amounts of aggregates are formed even under normal storage conditions. Fisher and Porter[12] examined highly purified insulins and the effect of static storage (−20 to 50°C) on the formation of high-molecular-weight polymers. They reported a decrease in biologic activity of approximately 3% after storage for 3 mo at 37°C, which was attributed primarily to the formation of high-molecular-weight polymers of insulin and desamido insulin. These polymers alone demonstrated no biologic activity in mouse convulsion assays.

In recent work, Thurow[13] examined the effect of rotational motion on the aggregation behavior of highly purified zinc and zinc-free insulins at neutral pH (40–100 U/ml). In these

motion studies, formulations containing potential stabilizing agents formed turbid gels in 4–27 h with the exception of one formulation containing 28 μg zinc/ml, which became turbid in 5 days. The addition of antioxidants had little effect on the aggregation behavior. He postulated that both high surface tensions present at the air-insulin interfaces and solid-liquid hydrophobic interactions were responsible for the rapid aggregation observed. Motion would indeed accelerate the exchange rate of insulin at these interfaces.

Further to previous work,[10] we here report the results of stability studies conducted with selected additives for protracted periods of up to 150 days. Conditions were chosen that would simulate the most severe encountered in an implantable delivery system. Nonionic, cationic, and ionic detergents (both physiologic and synthetic) were tested as stabilizers in view of their known protein-solvation characteristics and their potential to constrain the conformation of insulin[14] and other proteins in aqueous solution. Nonpolar solvents were added to formulations with the intent of reducing those hydrophobic, intermolecular interactions, which are known to play a major role in the dimerization of insulin. Physiologic compounds and extracts, some of which had previously been shown to dissolve zinc insulin crystals at neutral pH,[15] were similarly examined. The effect of various "medical grade" pump materials on the stability of these formulations was also investigated.

METHODS

Stock solutions containing (200 U/ml) insulin were prepared by dissolving recrystallized porcine insulin (Connaught Laboratories, Willowdale, Ontario, Canada) in distilled water adjusted to pH 2.0–2.5 with HCl. These were subsequently titrated to pH 7.0–7.4 with NaOH and stored for up to 1 wk at 4°C. Potential stabilizers were dissolved in distilled water at neutral pH to which stock insulin was added to a concentration of 5 U/ml. All formulations were prepared in duplicate and insulin crystals from the same lot were used throughout the study. To serve as controls, 20 samples of 5 U/ml insulin containing no additives were prepared as described above. U100 and U500 insulins with and without preservatives (0.2% m-cresol, 0.2% phenol) were also prepared in a similar manner. Final pH adjustment of all formulations was to 7.4 (with HCl or NaOH) unless otherwise specified.

All studies, excepting those in polyvinyl chloride (PVC) or silicone rubber containers, were carried out in 50-ml S18B glass vials fitted with S96-69 oxyglazed stoppers and 20-20 metal seals. Vials were labeled by a numerical code to ensure objectivity during examination of the solutions. Detergents, alcohols, and other additives were purchased from J. T. Baker, Sigma, Fisher Scientific, Merck and Co., Difco Labs Inc., Caledon Labs Ltd., or BDH.

Tests on compatibility of U5 insulin with selected pump materials were carried out by inserting cleaned material samples into the vials (duplicates). Materials examined were titanium, copper, polysulphone, cellulose acetate, and red, white, radio-opaque silicone rubbers (see Table 1 for traceable specifications). Material compatibility studies with concentrations of insulin higher than U5 were not conducted because of the insulin costs. Duplicate controls not containing insulin were run for each material in combination with each of the diluents investigated. These included m-cresol (0.2%) or phenol (0.2%).

After sterilization (steam autoclave) vials were filled through 0.22-μm filters (Millex-GS, Millipore, Bedford, Massachusetts) with 25 ml of formulation. Duplicates of solutions so prepared were subjected at 37°C to either: (1) shaking at 130 cpm in the horizontal plane (Eberbach reciprocal shaker) or (2) rotation at 20 cm from the axis of a wheel rotating at 60 rpm in the vertical plane. These conditions were chosen so as to simulate the most severe conditions that would be encountered in an implanted delivery system.

Solutions were visually examined at 4-day intervals to determine the degree of induced aggregation, which was visually assessed on a scale ranging from 0 (clear) to 10 (extreme turbidity). This scale was calibrated by assigning a range of percent transmittance at 540 nm to each value from 0 to 10. Optical density/% transmittance was measured on a Perkin Elmer Coleman 44 linear spectrophotometer. Formulation stability (FS) for each preparation subjected to motion was arbitrarily defined as the length of time over which percent transmittance remained above or equal to 96%. Formulations that contained visible aggregates were removed from the spinning/shaking apparatus, examined microscopically, and a random selection tested for microbial contamination. Those formulations with percent transmittance of >96% (visual code '0') after 3 wk of motion were examined at weekly intervals thereafter by light microscopy at 40, 100, and 250 ×

TABLE 1
Specifications of the materials tested for compatability with insulin formulations

Material	Specifications and uses
Copper	Pure copper—supplied by Metal Bellows, Infusaid Div., Sharon, Massachusetts
Titanium	Commercially pure titanium, passivated by soaking in nitric acid, rinsing with water followed by Freon MF and then Freon TF. Rinsed with distilled water before use in testing
Silicone rubbers	
Silastic, clear	Dow Corning medical grade silicone rubber tubing (0.035" × 0.065", ID × OD) (Dow Corning Canada Ltd., Streetsville, Ontario)
Silastic, red	*O-ring seals.* Food grade silicone compound. Supplied by: (1) Precision Associates, Minneapolis, Minnesota; (2) Parker Seal Co., Lexington, Kentucky
Silastic, white	Dow Corning medical grade silicone containing 9% barium sulfate
Polysulphone	0.005" × 0.015" (ID × OD) tubing as supplied by Union Carbide, Oakville, Ontario
Cellulose acetate	Membrane used as bacterial filter. Cellulose acetate 0.22-μm pore membrane (Millipore, Mississauga, Ontario)
Polyvinylchloride	4R2014 transfer pack material (Fenwal Labs., Div. of Travenol Labs Inc., Deerfield, Illinois)

magnification, and the pH of each measured. Those that remained stable for a prolonged period (FS >100 days) were checked by radioimmunoassay (RIA) to ensure that the clear solution was not an artifact resulting from adsorption of insulin onto the surface of pump materials and/or the wall of the test vessel.

Amicon UM05 ultrafilters were used to produce the <500 MW filtrates of bovine serum and of acid alcohol extracts of bovine pancreas.

In addition, the stability of commercially available U100 and U500 insulins from three manufacturers was also examined in triplicate. These were tested in their respective unopened vials.

RESULTS

The formulation stabilities (FS) of solutions subjected to either continuous rotation (FSR) or shaking (FSS) and containing the additive(s) listed are presented in Tables 2–6. Results were assessed in terms of enhanced stability (i.e., inhibition of aggregation) by comparison with 5 U/ml insulin in distilled H_2O. The latter demonstrated markedly poor stability with FSR values of 3–5 days (continuous rotation, 10 replicates) and 4–8 days (continuous shaking, 10 replicates). In comparison, formulations identical to the U5 insulin controls, but at concentrations of U100 and U500 had FSRs of 12 and 15 days, respectively (see formulation C, Table 2). FSRs for the U100 or U500 formulations were not increased by the addition of 0.2% m-cresol (Table 2). We had previously observed improved stability with another preservative, benzyl alcohol. Commercial preparations from four major manufacturers were diluted to 5 U/ml in distilled water and similarly tested. All aggregated in 4–10 days (data not shown). Two U100 commercial preparations were tested undiluted in their original vials. FSRs of 2 days and 36–46 days were obtained for products "A" and "B", respectively (see Table 2). The far less stable product "A" contained phosphate buffer and 0.3% m-cresol; product "B" contained 0.2% m-cresol and 1.6% glycerol and demonstrated marked stability.

Recently, Hoechst has developed an anti-aggregating insulin, Hoechst 21 PS, which contains a polypropylene-polyethylene glycol detergent. Five unopened ampoules of this U100 insulin yielded prolonged FSRs of 40–60 days. It is interesting that these results are in apparent disagreement with those reported by Hoechst (Dr. U. Grau, Hoechst Aktiengesellschaft, private communication), who observed no aggregation in over 100 ampoules that had been rotated for >1 yr. Test conditions are presently being compared in an effort to account for the discrepancy.

For each of the nonionic and anionic detergent formulations listed in Tables 3 and 4, controls were run without insulin, with and without the pump materials listed in Table 1. After 50 days of rotation, no reduction in percent transmittance was observed in any of the controls. The same was true of the controls for formulations listed in Tables 5 and 6 (no insulin, with and without pump materials). Controls were not run for a few of the formulations listed in Tables 5 and 6 and these are duly noted. Controls containing either m-cresol or phenol (at 0.3% and 0.2%, respectively) with and without pump materials also showed no reduction in percent transmittance for the duration of testing (50 days).

The stabilities of formulations containing nonionic and anionic detergents are shown in Tables 3 and 4. As is evident from the FS values, aggregate formation was inhibited by the nonionics; Brij 35 (0.1% vol/vol), Lubrol WX (0.1% vol/vol), Triton X 100 (0.02% vol/vol), Tween 20 (0.01% vol/vol), Tween 80 (1% vol/vol), and the anionic; SDS (0.05% wt/vol in 0.9% NaCl) and SDS (1% wt/vol). FSR values for these solutions were respectively 141, 155, 67, 68, 48, 80, and 150 days (rotational motion) as compared with 10 days for the insulin controls. Notably, the stability of the preparation containing 1% SDS was unaffected by all pump materials tested and remained clear for the duration of testing (150 days). However, all other detergent preparations when rotated in the presence of typical pump materials (see Tables 3 and 4 and material specifications, Table 1) showed markedly reduced stabilities. The remainder of the insulin-detergent formulations listed above when tested with silastic (red, white, clear), titanium, polysulphone, teflon, cellulose acetate, or copper (individually or as a group) yielded FSR values of 2–41 days.

TABLE 2
Stability of U100 and U500 formulations including bacteriostatic agents

Name	Concentration (U/ml)	Additives (%)	Formulation stability (days) FSR
A*	100	0.3% m-cresol, phosphate buffer	2
B*	100	0.2% phenol, 1.6% glycerol	36–46
C†	100	0.2% m-cresol	<12
C	100	0.2% m-cresol	<12
C	100	—	<12
C	500	0.2% phenol	<15
C	500	0.2% m-cresol	<15
C	500	—	<15
Hoechst 21PS‡	100	§	40–60

*Commercial preparations.
†Prepared from the same lot of crystals as U5 controls.
‡Tested in unopened native ampoules.
§A polypropylene-polyethylene glycol in lower concentration.

TABLE 3
Stability of insulin formulations with additives in the presence of pump components

Nonionic detergents			Formulation stability (days)	
Name	Concentration (vol/vol)	Components	FSR	FSS
Brij 35	0.1%	—	141	
	0.1%	Copper	(p40), 118	150
	0.1%	Titanium	(p22), 70	150
	0.1%	Teflon	40	150
	0.1%	Polyvinyl chloride	—	26
	0.1%	Silastic, clear	36	—
	0.1%	Polysulphone	40	150
	0.1%	Silastic, red	22	100
	0.1%	Silastic, white	28	87
	0.1%	All the above	25	—
Lubrol WX	0.001%	—	18	60
	0.01%	—	(p25), 155+	(p12), 60+
	0.01%	Teflon	8	(p7), 100+
	0.01%	Titanium	(p8), 8	(p7),100+
	0.01%	Polysulphone	19–51	19–61
	0.01%	Silastic, red	6	19
	0.01%	Silastic, white	2	2
	0.01%	Acetate	—	44
	0.01%	Copper	19	—
	0.1%	All the above	6	2
	0.1% + 1% BA		85	
	0.1% + 1% BA	Copper	14–25	
	0.1%	Silastic, clear	12–15	
Triton X100	0.02%	—	(p11), 67	74
	0.002%	—	4	48
	0.000002%	—	4	11
	0.1%	Silastic, clear	5	—
	0.1%	Silastic, red	9	—
	0.1%	Silastic, white	9	—
Tween 20	0.01%	—	(p12), 68	(p22), 81
	0.01%	Teflon	8	(p2), 19
	0.01%	Silastic, red	6	16
	0.01%	Silastic, white	2	2
	0.01%	Acetate	29	—
	0.01%	Titanium	29	(p2), 19
	0.01%	Polysulphone	29	(p2), 19
	0.01%	Silastic, clear	(p9), 15	—
	0.01%	All the above	2–6	2
	0.001%	—	12	60
	0.000001%	—	4	50
Tween 60	0.5%	—	3	50
	0.05%	—	19	10
	0.005%	—	7	19
	1% + 1% BA	—	25	—
Tween 80	1%	—	48	
	0.01%	—	8	84–88
	0.00001%	—	19	(p48), 162
	0.000001%	—	4	57

Note: (p) indicates presence of particles in an otherwise clear solution, (+) indicates that the trial was stopped before percent transmittance fell below 96; BA = benzyl alcohol.

With the exception of the 1% SDS solution, the stability of these detergent-insulin formulations was most severely and repetitively reduced in the presence of silicone rubber (FSR <10 days). The remainder of the materials examined had pronounced but extremely variable effects on stability of the solution. For example, the addition of titanium to the Brij 35 solution resulted in a 50% decrease in FSR whereas titanium in combination with the Lubrol formulation resulted in a 95% reduction (FSR). For both nonionic and ionic detergents, FSS values were 4.3 ± 1.7 (mean ± SEM) times higher than corresponding FSR values. This increased rate of aggregation in spun versus shook samples was observed in all but 3 of the 30 preparations listed in Tables 3 and 4.

As shown in Table 3, the nonionic detergent Tween 60 did not reduce the rate of aggregate formation. Tween 80 at concentrations less than 1% (vol/vol) did not significantly increase FSR values, but FSS was increased to 57 days at concentrations as low as 1×10^{-6} (vol/vol). At 1% (vol/vol) the FSR for Tween 80 was increased to 48 days.

As illustrated in Table 4, FSR values for the anionic bile salts sodium taurocholate (0.000002–0.02% wt/vol) and sodium deoxycholate (0.000002–0.02% wt/vol) were not sig-

TABLE 4
Stability of insulin formulations with additives in the presence of pump components

Anionic detergents			Formulation stability (days)	
Name	Concentration (vol/vol)	Components	FSR	FSS
Sodium dodecyl	0.05%	—	6	6
Sulphate	0.05%	Silastic	6	
	0.05%	SDS washed, H_2O rinsed, silastic	9	
	0.05%	SDS washed silastic	9	
	0.05% in 0.9% NaCl		(p69), 80+	(p3–5), 200+
	0.05% in 0.9% NaCl	Silastic	14	
	0.05% in 0.9% NaCl	SDS washed, H_2O rinsed, silastic	6–11	
	0.1%	Silastic	9	
	0.1%	Silastic, red	12	
	0.1%	Silastic, white	41	
	1%		(p26),150	
	1%	All parts separately	(p26), 150	
Sodium taurocholate	0.02%	—	(p4), 8	
	0.0002%	—	4	(p48), 67
	0.000002%	—	4	15
	0.02% + 1% BA	All parts separately	10	
	1%	All parts separately	0	
Sodium deoxycholate	0.02%	—	4	134
	0.0002%	—	4	4
	0.000002%	—	4	(p11), 19

Note: (p) indicates presence of particles in an otherwise clear solution; (+) indicates that the trial was stopped before percent transmittance fell below 96; BA = benzyl alcohol.

nificantly greater than the insulin controls (FSR <10 days). The apparent success of the anionic detergent, sodium dodecyl sulphate (SDS) at 0.05% wt/vol in 0.9% NaCl led to the testing of this formulation in combination with pump materials. This formulation, when rotated in the presence of silicone rubbers, gave FSRs of up to 14 days. It was postulated that either these rubbers had released a factor(s) that promoted aggregation or that the effective concentration of SDS had been reduced by absorption of the detergent into the rubber. For this reason, experiments in which the silastic was presoaked with SDS and then rinsed with distilled water before testing were carried out. No advantage was noted

TABLE 5
Stability of insulin formulations with additives in the presence of pump components

Physiologic compounds and extracts			Formulation stability (days)	
Name	Concentration	Components	FSR	FSS
Histidine	0.1 M	—	7–11	
Dehydroascorbic acid†	0.01 M	—	6	
Hyaluronic acid†	0.2% + 1% BA	—	6	
n-Acetyl neuraminic acid	0.2%	—	6	
Glutamic acid*	0.01 M + 1% BA	—	5	
		All parts separately	5	
	5%	All parts separately	6	23
Lysophosphatidyl choline (Myristol)	0.02% vol/vol in 20% ethanol	—	68	
Lysophosphatidyl choline (egg yolk)	0.02% vol/vol in 20% ethanol	—	47	
500 MW filtrate of acid alcohol extract of pancreas	25% vol/vol in 0.01 M histidine			p40
	25% vol/vol in 0.1 M phosphate		p40	"
	5% vol/vol in 1% BA	—	40+	
	5% vol/vol in 1% BA	Silastic, clear	40+	
500 MW filtrate of bovine serum	4% vol/vol	—	4	15

Note: (p) indicates presence of particles in an otherwise clear solution; (+) indicates that the trial was stopped before percent transmittance fell below 96; BA = benzyl alcohol.
*Initial pH = 3.8; final pH = 3.18.
†Control without insulin not run.

with this pretreatment. Concentrations of 1% SDS were required to maintain FSR values of >150 days when silicone rubbers were present.

A limited number of cationic detergents tested, such as trimethyl ammonium bromide, caused precipitation of insulin during or a few hours after preparation of the formulation.

Some physiologic compounds and extracts were examined in stability testing as reported in Table 5. Glutamic acid (0.1 mol/L), which at its isoelectric point has been claimed to decrease aggregate formation,[16] yielded FSRs of 7–11 days. At various concentrations up to 50 mM, proline, phenylalanine, tryptophan, glycine, histidine, n-acetyl neuraminic acid, hyaluronic acid, dehydroascorbic acid, threonine, and isoleucine were ineffective.

While the <500 MW filtrate of bovine serum was ineffective as a stabilizer, FSRs of 40 days were obtained with the <500 MW filtrate of bovine pancreas containing approximately 20% ethanol. The weakly chelating buffers, sodium phosphate (0.1–0.002 mol/L) and histidine (0.1 mol/L), although remarkably increasing the solubility of zinc insulin at neutral pH, resulted in FSRs of <18 days.

The physiologic detergent lysophosphatidylcholine was ineffective when employed at levels present in human serum (0.0007% wt/vol). However, at 0.02% (in 20% ethanol), the extracted and synthetic lysophosphatidylcholines (Sigma Chemicals, St. Louis, Missouri) increased FSRs to 47 and 68 days, respectively (Table 5).

As shown in Table 6, with the alcohols [benzyl (1%), isopropanol (10–50%), and glycerol (5–50%)], stabilization for periods of up to 60 days were obtained in the absence of pump materials. Glycerol and isopropanol in concentrations of 30–50% and 10–50%, respectively, resulted in a high degree of stabilization with FSR values between 40 and 60 days. Benzyl alcohol at 1% was the least effective of these

TABLE 6
Stability of insulin formulations with additives in the presence of pump components

Salts, buffers and alcohols			Formulation stability (days)	
Name	Concentration (vol/vol)	Components	Spin	Shake
Benzyl alcohol (BA)	1%	—	20	21
	1%	Silastic, clear	26	
Acetonitrile*	10%	—	—	10–30
Ethanol*	20%	—	10	—
Glycerol	5%	—	(p31), 80	
	10%	—	13	
	30%	—	40–60	
	50%	—	(p40), 60	
	50%	—	p40	
	50%	Silastic	5	
	50%	Silastic, white	5	
	50%	All parts separately	5	
Isopropyl alcohol				
	5%	—	14	
	5%	Silastic	14	
	10%	—	50	
	10%	Silastic	7	
	20%	—	50	
	20%	Silastic	14	
	50%	—	50	
	50%	Silastic	14	
Polyethylene glycol	0.2%	—	3	
	2%	—	6	
	10%	—	6	
	20%	—	6	
Sodium chloride*	0.9%	—	5	5–7
Sodium phosphate*	0.1 M	—	11	
	0.02 M + 1% BA	—	11	
	0.002 M + 1% BA		18	
Sodium bicarbonate with acetic acid and sodium acetate	0.025 M 0.02 M 0.1 M		3–17	
Sodium bicarbonate with sodium phosphate and sodium citrate	0.025 M 0.1 M 0.01 M		11–13	
Sodium bicarbonate with oxalo-acetic acid	0.025 M 0.01 M		17–20	
Sodium bicarbonate with 1% BA	0.0012 M		5–11	
		Copper	20–60	
		All parts separately	5–11	

Note: (p) indicates presence of particles in an otherwise clear solution; (+) indicates that the trial was stopped before percent transmittance fell below 96; BA = benzyl alcohol.
*Control without insulin not run.

alcohols. Silicone rubbers suitable for application in medical delivery systems produced rapid aggregate formation in both the glycerol and isopropanol preparations (FSR ≤ 14 days). However, the 1% benzyl alcohol formulation had an FSR of 20 days in the absence of and 26 days in the presence of silicone rubber. Polyethylene glycol at 0.2–20% vol/vol did not significantly increase formulation stability.

Formulations in 25 mM sodium bicarbonate with phosphate-citrate or oxaloacetate buffers demonstrated mildly increased stability with FSRs of 11–20 days. Reduced FSRs of 5–11 days were obtained with 1.2 mM sodium bicarbonate in combination with 1% benzyl alcohol. Interestingly, the rotation of this formulation in the presence of copper wire resulted in improved, albeit variable, stability (FSR = 20–60 days).

Analysis of insulin aggregates formed during these rotation/shaking studies was encumbered by their low solubility in: HCl (5 mol/L), urea (8 mol/L or 6 mol/L in TRIS at pH 9), benzene, absolute alcohol, and SDS (0.1–1%). Toward the end of the study, it was observed that aggregates so formed may be totally resolved in 6 M guanidine at pH >10 5 or in 9:1 phenol/H_2O (partial solution at pH >10.5 in H_2O). Results of the analysis of such aggregates have recently been reported.[17]

DISCUSSION

We previously reported[10] the results of preliminary stability studies in a review of factors known to promote insulin aggregation. In the present study, the method of stability testing was modified and a large variety of additives to insulin, many of them detergent in nature, were assessed alone or together with some of the materials used in insulin delivery systems. It should be stressed that the motion studies here were conducted under severe conditions in an attempt to elucidate factors responsible for inhibiting (or promoting) the aggregation of insulin. Therefore, the results indicate only in general which classes of formulations or materials warrant further investigation. Interfaces (solid-liquid, gas-liquid) are additional promoters of aggregation (unpublished data) and their effects have been accentuated here in contrast to the usual pump situation where large air volumes are not included.

In these studies, rotation proved to be a much more severe means of testing stability. On the average, formulation stabilities under conditions of rotation (FSR) were four times lower than formulation stabilities under conditions of reciprocating linear motion (FSS). This would indicate that spreading of insulin monomers or polymers thereof across gas-liquid or solid-liquid interfaces may be important parameters in the aggregation process. Many formulations in the latter half of the study were thus subjected only to rotation.

A direct relationship between insulin concentration and stability was observed for the zinc porcine neutral insulins containing no additives. Formulations of U100 and U500 were, respectively, 2–3 and 3–5 times as stable as U5 preparations (formulated from the same lot). As we have previously reported, HMWP formations involve the non-covalent binding of insulin monomers[17] and it has been proposed that the initial step involves partial unfolding of the tertiary structure.[13] In such a process, the concentration of unaltered monomer would be expected to competitively inhibit the self-association of denatured monomers and would account for the positive relationship between concentration and stability observed.

With respect to the stabilizers employed, it is apparent that all the anionic and nonionic detergent additives, with the exception of Tween 60, markedly increased the stability of their respective formulations when these were subjected to continuous rotation at 37°C.

As the effect of micelle formation on insulin solutions was unknown, detergents were tested at concentrations ranging both above and below their predicted critical micellar concentrations (CMC).[18] Upon examination of the detergent group as a whole it is apparent that FSRs were increased when detergent concentrations were at or near their CMC (0.01–0.1%).[19] Higher concentrations than these were in general not tested in view of their reported hemolytic and toxic effects. Detergent concentrations of 0.01–0.001% were individually chosen so as to give an approximately equimolar concentration of detergent to insulin. Detergent concentrations lower than this equimolar value failed to increase formulation stability significantly. However, the low concentration of the polypropylene-polyethylene glycol used in Hoechst 21 PS appears sufficient to increase stability 3.5–5-fold over U100 CZI solutions (Table 2, manufacturer C), but proved only slightly more stable than the insulin obtained from manufacturer B (see Table 2), which contained phenol.

Of the nonpolar solvents, glycerol at 5–50% and isopropyl alcohol at 10–50% were moderately effective stabilizers, but the degree of stabilization was less than that seen with the detergent group as a whole. The addition of as little as 1.6% glycerol and 0.2% phenol to the U100 CZI formulations increased stability from 12 to >40 days (FSR). This perhaps explains in part the discrepancy observed between the stabilities of the three U100 commercial preparations tested. The preparation from manufacturer A contained m-cresol and phosphate buffer (FSR = 2 days) and from B contained phenol and glycerol (FSR = 40 days). These differences may also be attributable to variations in zinc concentration (appropriately elevated zinc concentrations have been shown to favor stability[13] and phosphate is known to reduce the amount of zinc bound at neutral pH).

The improvements in stability obtained with the alcohols and the detergents were lost when these formulations were rotated in the presence of selected pump materials. Almost without exception FSRs were drastically reduced. The silicone rubbers produced the most dramatic decreases in FSS and FSR values, up to 95% in some instances. In this respect, it would appear that SDS concentrations much greater than the CMC are required to maintain equal FSRs in the presence of silicone rubbers.

The effects on stability of the materials other than silicone rubber were extremely variable. It would appear that the degree to which any one of these remaining materials destabilizes one formulation cannot be correlated to the rest of the group. The most probable mechanism would involve very specific binding of additive to some material surfaces and not others. Inhibition of aggregation solely by competitive binding to portions of the insulin molecule would not be expected to produce such diversified results.

The small, anionic bicarbonate molecule at concentrations of 25 mM mildly enhanced the stabiltiy of insulin solutions. This ion was previously found to promote the dissolution of

insulin crystals at neutral pH.[15] Other molecules with similar dissolution properties, such as histidine and imidazole, did not increase formulation stability. It is known that solubility may be enhanced by chelation of Zn^{2+} from the hormone and that both zinc and zinc-free insulins demonstrate concentration-dependent reversible aggregation behavior at neutral pH.[20] This reaction, however, is shifted toward the monomer with the removal of certain divalent metal ions, notably Zn. In view of these findings, one might have expected chelating reagents to have reduced aggregation in our previously reported results.[10] It was observed, however, that neither the strong chelator EDTA, nor weak chelators (citrate and histidine) were effective stabilizers. In those studies and in current unreported tests using EDTA (10 mM) in conjunction with other additives, it would appear that EDTA decreases formulation stability. This is in agreement with those studies that indicate increased stability with appropriate levels of Zn[13] or Ca.[21]

Of the physiologic detergents, the glyceride lysophosphatidylcholine remarkably increased stability while the bile salts were ineffective. Unfortunately, lysophosphatidylcholine, in addition to its prohibitive cost, required the addition of 20% ethanol (or other organic solvents) for solubilization. This rendered the formulation unphysiologic unless delivered in small doses. In addition, the increased stability of other formulations containing ethanol alone suggests that stabilization may not have been solely due to the action of lysophosphatidylcholine.

Several mechanisms for the observed enhancement of stability can be postulated. The most obvious is a reduction in the polarity of the effective environment of the insulin molecule. Alcohols such as isopropanol and glycerol reduce the polarity of the entire medium. On the other hand, detergent-type molecules, composed of long hydrocarbon moieties with or without ionic end groups, form hydrophobic enclaves (micelles) in which the insulin molecule could be sheltered from the surrounding hydrophilic medium and stabilized in an ordered conformation.

In this respect, Wu and Yang[14] have studied the effects of various detergents on the CD (circular dichroism) spectrum of insulin. They observed that the CD spectrum of insulin in SDS (25 mM, 0.72%) resembled that of des (23–30) octapeptide insulin. Insulin dimers are held together by an antiparallel, hydrogen bonded, B-form structure between the B23–29 residues at the carboxy termini of the B chains. Des (B23–30) octapeptide insulin cannot, therefore, form dimers. Thus, Wu and Yang[14] attributed the change in the CD spectrum of native insulin seen with the addition of SDS to the disruption of dimers. The stabilization observed in our studies may well be the result of this stabilization of the monomer. Wu and Yang[14] also showed that nonionic polyoxyethylene ether detergent, $C_{12}E_7$, (Nikko Chemicals, Japan) had little effect on the CD spectrum of insulin, indicating that the dimer form was not disrupted. Our partial success with the polyoxyethylene-type detergents (Brij, Tween, Triton) might therefore be attributed to stabilization of the dimer or higher polymers rather than the monomer.

In both nonionic detergents and alcohols the conformation of the protein may be constrained to minimize exposure of polar side-chains to the less polar environment. It may indeed be that the aggregation process described in this study is similar to that for insulin fibrils. The latter may be formed by denaturation with heat (or by extrusion) and are reported to result from (partial) uncoiling of the protein during which intrachain hydrogen bonds between amino and carboxyl groups are replaced by interchain hydrogen bonds.[22] Shielding of the polar side-chains by detergents or alcohols should reduce the possibilities of such intermolecular bonding. It is indeed interesting that both insulin fibrils (produced by methods previously reported[22]) and those aggregates produced in this study are resolved in media that disrupt hydrogen bonds, i.e., high concentrations of phenol, guanidine-NaOH, or extremely alkaline pH (partial dissolution only).

It is also possible that both the alcohols and detergents are acting to reduce hydrophobic associations between phenolic side-chains. The importance of these in the dimerization of insulin is well known.[23] In studies with [tetrakis (3-nitrotyrosine)] insulin, Carpenter et al.[24] demonstrated that above the pKa of the nitrotyrosine phenolic group (pKa = 7.3), this insulin did not aggregate (sedimentation equilibria experiments). Their results thus implicate protonated tyrosyl residues in the aggregation process, the normal pKa of the phenolic group being 10.4. However, caution must be exercised in correlating these results to the type of aggregation induced by motion at elevated temperatures in our experiments.

It is tempting to speculate that the effect of motion per se or motion across interfaces with high surface tensions is to uncoil partially the globular insulin molecule and expose portion(s) of its hydrophobic core. In this respect both the detergents and alcohols may be serving a dual purpose in reducing hydrophobic interactions between denatured insulin molecules and in reducing the surface forces acting on the hormone.

In summary, it may be said that molecules that stabilize insulin formulations have been found. The alcohols and detergents discussed here are, however, required in such concentrations that the resulting formulations may be unphysiologic in anything but very low doses. Although the aggregation process is not clearly understood, studies must continue on the analysis of aggregated material and the interaction of insulin with surfaces with the hope not only of understanding but also of preventing the reactions involved.

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TI Varying effects of cyclodextrin derivatives on aggregation and thermal behavior of ***insulin*** in aqueous solution
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Varying Effects of Cyclodextrin Derivatives on Aggregation and Thermal Behavior of Insulin in Aqueous Solution

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Maltosyl-β-cyclodextrin (G_2-β-CyD) suppressed the aggregation of insulin in neutral solution, while the sulfate of β-CyD (S-β-CyD) accelerated the aggregation. On the other hand, the sulfobutyl ether of β-CyD (SBE-β-CyD) showed varying effects on insulin aggregation, depending on the degree of substitution of the sulfobutyl group: *i.e.*, the inhibition at relatively low substitution and acceleration at higher substitution. Differential scanning calorimetric studies indicate that the self-association of insulin stabilized the native conformation of the peptide, as indicated by an increase in the mean unfolding temperature (T_m). G_2-β-CyD and SBE-β-CyD decreased the T_m value of insulin oligomers, while S-β-CyD increased the T_m value. ^{1}H-Nuclear magnetic resonance spectroscopic studies suggest that G_2-β-CyD includes accessible hydrophobic side chains of insulin within the CyD cavity, and hence perturbs the intermolecular hydrophobic contacts between aromatic side chains across the monomer–monomer interfaces. By contrast, the electrostatic interaction between the positive charges of insulin and the concentrated negative charges of the sulfate and sulfonate groups of the anionic β-CyDs seems to be more of a factor than the inclusion effects. These results suggest proper use of the CyD derivatives could be effective in designing rapid or long-acting insulin preparations.

Key words bovine insulin; cyclodextrin derivative; inclusion complexation; aggregation; differential scanning calorimetry; ^{1}H-NMR spectroscopy

The propensity of insulin to form reversible and irreversible aggregates in solution is of great concern as it may lead to the loss of biological potency, immunogenic reactions, blockage of infusion pumps or an unacceptable physical appearance in long-term therapeutic systems.[1] In the neutral solutions used clinically, insulin is mostly assembled as a zinc-containing hexamer and its self-association limits the rate of subcutaneous absorption, which is too slow to mimic the normal rapid increase of insulin in blood at the time of meal consumption.[2] These problems are further complicated by the tendency of insulin to adsorb onto the hydrophobic surfaces of containers and devices, perhaps by mechanisms similar to those inducing aggregations.[3] To overcome these drawbacks, several approaches have been proposed, including the use of amphiphatic excipients,[4] chemical modification[5] and site-directed mutation.[6]

An alternative to these strategies is complex formation with cyclodextrins (CyDs).[7–9] Our previous studies have shown that some hydrophilic CyDs, including maltosyl-β-CyD (G_2-β-CyD) and 2-hydroxypropyl-β-CyD, significantly inhibit the adsorption of bovine insulin to hydrophobic surfaces of containers and its aggregation by interacting with hydrophobic regions of the peptide.[10] Recently, the sulfates and sulfoalkyl ethers of CyDs have been evaluated as a new class of parenteral drug carriers because they are highly hydrophilic and less hemolytic than the parent and the other hydrophilic CyDs.[11,12] Following up on these studies, the effects of anionic β-CyD derivatives on the aggregation and thermal behavior of insulin in solution both at acidic and nearly neutral pHs were investigated and compared with that of G_2-β-CyD, with emphasis on the contribution of their inclusion ability and polyanionic character to the interaction with the peptide.

Experimental

Materials Bovine insulin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), lot No. 26H170, with a nominal activity of 28.1 I.U./mg (bovine pancreas crystalline, I-5500), and was used without further purification. The content of zinc ion was about 0.5%, corresponding to approximately 2 mol of zinc ion per 1 mol of insulin hexamer. G_2-β-CyD was a generous gift from Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan). SBE-β-CyDs with average degrees of substitution of 3.9 and 6.2 (SBE4-β- and SBE7-β-CyDs) were donated by CyDex L.C. (Overland Park, KS, U.S.A.). S-β-CyD with an average degree of substitution of 10.7 was prepared by a non-regional selective method as described previously.[13] The structures and abbreviations of CyDs used are listed in Table 1. All other materials were of reagent grade, and deionized double distilled water was used.

Aggregation Studies Insulin was dissolved in 10 mM sodium phosphate buffer (pH 6.8, $I=0.2$) in the absence and presence of additives. The aggregation of insulin was evaluated by measuring the insulin in the filtrate (filter: DISMIC-13CP045AN; Advantec Co., Tokyo, Japan) after freshly prepared insulin solutions stood in a silicone-coated glass tube at 25 °C; this container was used to minimize the adsorption of the peptide onto the surface. The concentration of insulin in the filtrate was determined by high-performance liquid chromatography (HPLC). The HPLC conditions were as follows: pump, LC-10AD (Shimadzu Co., Kyoto, Japan); detector, UVDEC 100 V-UV (210 nm, Jasco, Tokyo, Japan); column, YMC Pack C_8 AP-type, (4.6 mm i.d. × 150 mm; flow rate, 1.0 ml/min; 40 °C; YMC Co., Kyoto, Japan); internal standard, methyl *p*-aminobenzoate. The mobile phase consisted of 20% (v/v) acetonitrile and 0.05% (v/v) trifluoroacetic acid (TFA) in water as solvent A; 60% (v/v) acetonitrile and 0.05% (v/v) TFA in water as solvent B. A gradient profile: 0 to 100% B in 20 min, hold 100% B for 1 min, and return to initial condition immediately, was applied.

The volume–surface diameters of micron-sized insoluble insulin particles were measured using a Galai CIS-1 laser scan grading analyzer (Migdal Haemek, Israel). The distribution of submicron-sized soluble insulin oligomers was determined at 25 °C by quasielastic light scattering, using a submicron analyzer (NICOMP Model 370 EVHPL, Pacific Scientific Co., Palo Alto, CA, U.S.A.) equipped with 64 channels, a detector set at an angle of 90°, and an argon laser set at the blue-green 488 nm line, having a maximum power of about 280 mW (INNOVA 70, Coherent, Inc., Palo Alto, CA, U.S.A.). For each sample, light-scattering measurements were accumulated during about 10-min intervals to reduce random signal noise and to ensure a stable baseline.

Differential Scanning Calorimetry Scanning calorimetric measure-

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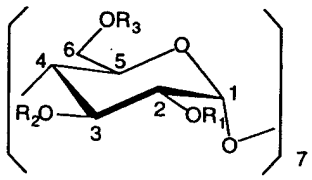
Table 1. Chemical Structures of Cyclodextrin Derivatives Used in This Study



Compound	Abbreviation	R_1	R_2	R_3	DS[a]
β-Cyclodextrin	β-CyD	H	H	H	—
6-*O*-Maltosyl-β-cyclodextrin	G_2-β-CyD	H	H	H or maltose	1
Sulfobutyl-β-cyclodextrin	SBE4-β-CyD	R_1, R_2, R_3 = H or $(CH_2)_4SO_3Na$			3.9
	SBE7-β-CyD				6.2
Sulfated β-cyclodextrin	S-β-CyD	R_1, R_2, R_3 = H or SO_3Na			10.7

a) The average degree of substitution.

ments were made using an MC-2 differential microcalorimeter (MicroCal, Inc., Northampton, MA, U.S.A.) using the MicroCal Origin for data acquisition and analysis. All solutions were degassed under a vacuum before being loaded into the calorimeter cells. The DSC scans were performed at a rate of 1 °C/min in the temperature range from 5 to 95 °C under an excessive N_2 pressure at about 220 kPa. The calorimetric enthalpies of thermal unfolding, accompanied by the dissociation of insulin oligomers in the absence and presence of CyDs, were obtained from the DSC recordings of excess heat capacity changes. After subtracting the reference buffer data, the raw data obtained in the form of heat capacity as a function of measuring temperature were converted to excess molar heat capacity using the scan rate and the peptide concentration.

Proton-Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy The ^{1}H-NMR spectra of insulin (2 mM) in the absence and presence of CyDs (0.5 mM for SBE-β-CyDs or 100 mM for G_2-β-CyD) were taken on a JNM EX-400 spectrometer (Jeol, Tokyo, Japan), operating at 399.65 MHz at 25 °C, using 30%(v/v) CD_3COOD as a solvent. ^{1}H-NMR chemical shifts were given in parts per million (ppm) relative to that of the HOD signal or the CD_3COOD signal, with an accuracy of ±0.001. The ^{1}H-NMR signals of the aromatic region of insulin were assigned according to the reports of Hua and Weiss[14] and of Funke and co-workers.[15]

Results and Discussion

Effects of CyDs on Insulin Aggregation in Solution In solution, insulin exists in an equilibrium between monomers, dimers, hexamers and higher order aggregates, depending on environmental factors such as concentration, pH, ionic strength, temperature and metal ions. The two insulin molecules in the dimer are held together by predominantly hydrophobic forces, reinforced by four hydrogen bonds arranged in an antiparallel β-sheet structure between the two C-terminal strands of the B chain. In the presence of zinc ions three insulin dimers are assembled into a hexameric organization, in which the zinc ions are coordinated to B10 histidines.[1]

In neutral solutions, insulin is mostly polymerized as a zinc-containing hexamer, eventually leading to the precipitation of higher order aggregates. A substantial aggregation of insulin occurred at concentrations of more than 0.05 mM in a phosphate buffer (pH 6.8, I=0.2) at 25 °C, as indicated by the increased optical density at 600 nm and the decreased concentration of the peptide remaining in the solution.[10] The HPLC analyses of the redissolved insulin aggregates revealed no fragmentation of the peptide and the non-covalent nature of the aggregates.

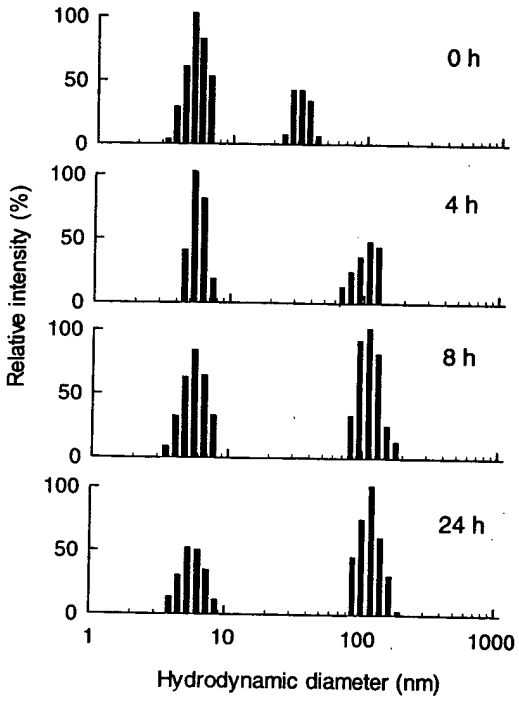


Fig. 1. Changes in Intensity-Weighted Distribution for Soluble Insulin Particles after Preparation of Insulin Solution (0.15 mM) in Phosphate Buffer (pH 6.8, I=0.2) at 25 °C

Figure 1 shows changes with time in intensity-weighted distribution for soluble insulin particles after preparation of the insulin solution (0.15 mM) at 25 °C, where micron-sized insoluble particles corresponding to highly aggregated insulin (>0.22 μm) were filtered out prior to analysis. A freshly-prepared insulin solution showed bimodal distribution of particles with mean hydrodynamic diameters of 5.3 nm and 31.2 nm, respectively. The first peak, at 5.3 nm in diameter, seems to be primarily an insulin hexamer, as judged by crystallographic dimensions for the hexamer, showing an almost cylindrical structure with a diameter of 5 nm and a height of about 3.5 nm.[1] The second peak, at 31.2 nm in diameter, may indicate intermediate assemblies, the diameter of which increased with the elapse of time and reached a critical size over 100 nm 24 h after preparation. Furthermore, the intensity ratio of the second peak to the first one increased from

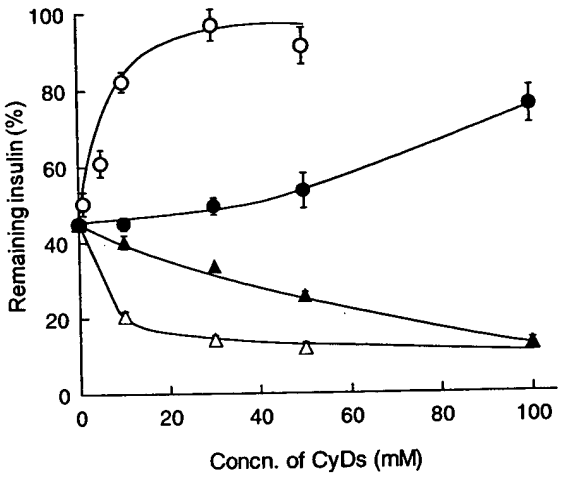


Fig. 2. Effects of CyDs on Aggregation of Insulin (0.15 mM) 24 h after Preparation of Insulin Solution in Phosphate Buffer (pH 6.8, $I=0.2$) at 25 °C, as a Function of CyD Concentrations

○, with G_2-β-CyD; ●, with SBE4-β-CyD; △, with SBE7-β-CyD; ▲, with S-β-CyD. Each point represents the mean ± S.E. of 4 experiments.

0.4 at time zero to 1.7, 24 h after preparation. Sluzky *et al.* have proposed a mathematical model describing the kinetics of insulin aggregation in aqueous solution upon agitation in the presence of hydrophobic surfaces, in which partially unfolded insulin monomers associate and form the initial nucleating species, eventually leading to the insulin aggregation.[16] In the same way, the intermediate species observed here would serve as precursors to micron-sized aggregates *via* successive kinetic processes.

Figure 2 shows the effects of CyDs on the aggregation of insulin 24 h after preparation of the insulin solution (0.15 mM) in phosphate buffer (pH 6.8) at 25 °C, as a function of the CyD concentrations. The CyDs did not affect the mean volume–surface diameter of insoluble insulin particles, which was determined to be 28.4 ± 2.6 μM. Unfortunately, the presence of the CyDs at higher concentrations made it impossible to determine the submicron-range particle distribution for insulin, because of the overlapping of extremely large scattering intensity peaks corresponding to the CyDs and their hydrates.[17] G_2-β-CyD suppressed the aggregation of insulin in a concentration-dependent manner. Under the present condition, a minimal concentration of G_2-β-CyD which was necessary to achieve statistically significant inhibition of insulin aggregation was 5 mM, at which the molar ratio of G_2-β-CyD to insulin was 33 : 1, and complete inhibition was observed at concentrations more than 30 mM. G_2-β-CyD may interact with hydrophobic amino acid residues of insulin, and thus prevent the aggregation by eliminating intermolecular hydrophobic contacts.[10] This view was confirmed by a dilution microcalorimetric study, in which CyDs increased the dissociation of insulin oligomers in a manner consistent with their binding to the dissociated form of the peptide molecule.[18] On the other hand, S-β-CyD accelerated the insulin aggregation. Since S-β-CyD has highly concentrated negative charges located near the entrance of the cavity and shows limited inclusion ability, its polyanionic character may contribute to the accelerated aggregation of insulin.

As shown in Fig. 2, SBE-β-CyD showed varying effects on insulin aggregation, depending on the degree of substitution; *i.e.* inhibition at relatively low substitution and acceleration at higher substitution. Since the sulfonate groups in SBE-β-CyD are appropriately spaced from the CyD cavity with a butyl chain and do not interfere with the inclusion process, SBE4-β-CyD may inhibit the insulin aggregation in a manner similar to G_2-β-CyD. In the case of SBE7-β-CyD, the electric effects seem to be more of a factor than the inclusion effects, eventually leading to the acceleration of the insulin aggregation.

Under the present condition with pH 6.8 higher than the isoelectric point of insulin ($pI=5.3$),[19] the net charge of the peptide is negative. The charged and polar groups on the insulin surface would be surrounded by water molecules *via* ionic hydration and/or hydrogen bondings. High concentrations of sulfates such as $(NH_4)_2SO_4$ and Na_2SO_4 are commonly used to precipitate or crystallize polypeptides in the native form, which can be ascribable to the loss of the hydration layer on the peptide surface.[20] In fact, some neutral salts accelerated the aggregation of insulin, with the efficacy increasing in the order: NaCl $\ll CH_3COONa < Na_2SO_4$, a sequence which corresponds to their salting-out potency.[21] For instance, under the same condition as in Fig. 2, the remaining percentages of insulin in the presence of 100 mM NaCl, CH_3COONa and Na_2SO_4 were 41.5 ± 1.9%, 22.9 ± 1.3% and 18.6 ± 1.0%, respectively. Therefore, the sulfate and sulfonate groups in S-β-CyD and SBE-β-CyD would remove the hydration layer from the insulin molecule in a manner similar to these lyotropic anions, a situation which makes the intermolecular interaction of the peptide stronger, eventually leading to the accelerated association or aggregation of the peptide. The logarithm of peptide solubility (S) is known to decrease almost linearly with the ionic strength (I) of salts according to the following equation[22]:

$$\log S=\beta-KsI \quad (1)$$

where β and Ks are empirical constants, Ks being the salting-out constant. The Ks values increased in the order: S-β-CyD (0.386) < Na_2SO_4 (0.736) < SBE7-β-CyD (0.952).

As another aspect of the effects of the anionic CyDs on the insulin aggregation, S-β-CyD and SBE-β-CyD are likely to induce a conformational transition in the insulin hexamer in a manner similar to lyotropic anions, which partially transform an extended chain from the B1 to B9 residues (T-state) into an α-helix (R-state) *via* electrostatic screening of an ion pair interaction between the B1-phenylalanine and the A17-glutamic acid at the dimer–dimer interface.[23] This may also contribute to the accelerated association of the peptide.

The varying effects of the CyD derivatives on the insulin association were confirmed by the ultrafiltration experiments shown in Fig. 3. G_2-β-CyD and SBE4-β-CyD facilitated the permeation of insulin through an ultrafiltration membrane with a nominal molecular weight cut-off of 50 kDa, the former being more effective. By contrast, S-β-CyD and SBE7-β-CyD reduced the membrane permeation of insulin, as reflected by the accelerated association of the peptide. Our previous studies have dem-

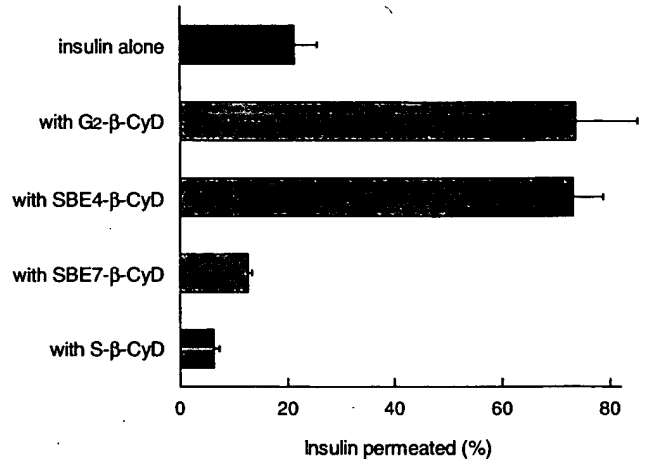


Fig. 3. Effects of CyDs (100 mM) on Permeation of Insulin (0.1 mM) through Ultrafiltration Membrane (XM 50) in Phosphate Buffer (pH 6.8, $I=0.2$) at 25 °C

Each point represents the mean ± S.E. of 2—9 experiments.

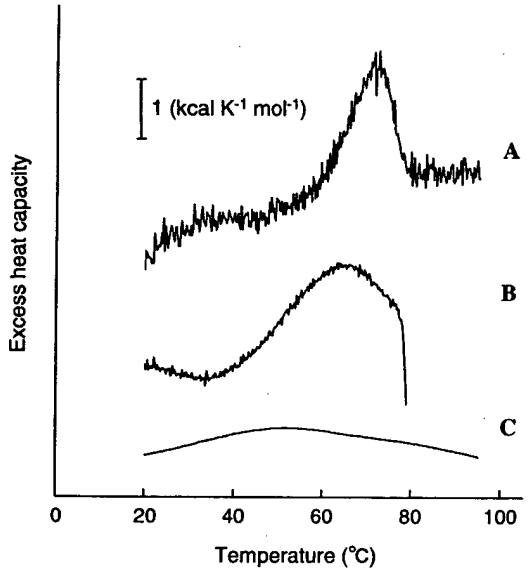


Fig. 4. Typical Excess Heat Capacity Curves for Thermal Unfolding of Insulin at Different Association States

A, insulin (0.1 mM) in phosphate buffer (pH 6.8, $I=0.2$); B, insulin (0.3 mM) in phosphate buffer (pH 2.0, $I=0.2$); C, insulin (5 mM) in 30% (v/v) acetic acid solution.

onstrated that the permeation of insulin mediated by G_2-β-CyD was much greater than that by ethylenediaminetetraacetic acid (EDTA), which is known to sequester zinc ions from insulin oligomers and dissociate them into the dimer.[24] Furthermore, G_2-β-CyD facilitated the permeation of insulin through the membrane in an acidic solution at pH 2.0, in which the peptide exists primarily as a zinc-free dimer.[10] These results indicate that G_2-β-CyD shifts the equilibrium in favor of the monomeric form.

Effects of CyDs on Thermal Behavior of Insulin Figure 4 shows the typical excess heat capacity curves for thermal unfolding of insulin at different association states, after base-line subtraction and concentration normalization. The concentration of insulin used was chosen here based on the solubility of the peptide at different association

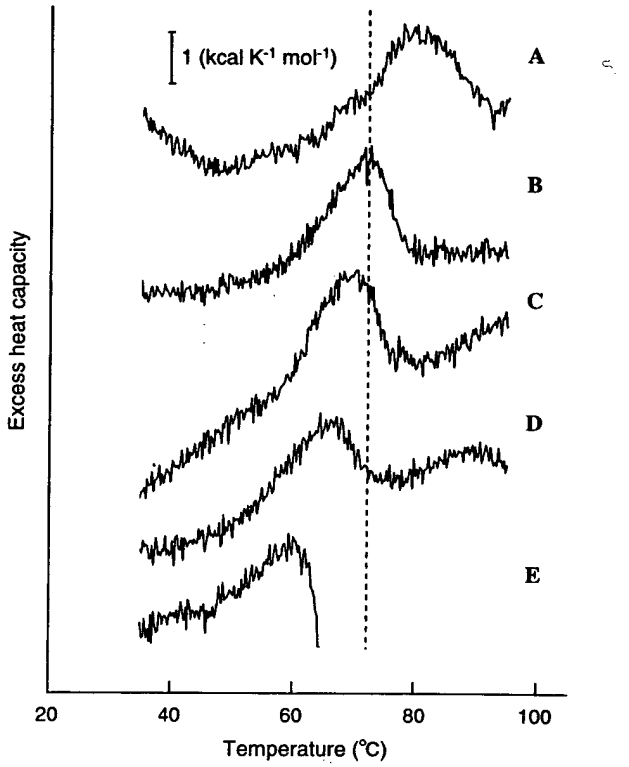


Fig. 5. Effects of CyDs (100 mM) on Excess Heat Capacity Curve for Thermal Unfolding of Insulin (0.1 mM) in Phosphate Buffer (pH 6.8, $I=0.2$)

A, with S-β-CyD; B, insulin alone; C, with SBE7-β-CyD; D, with SBE4-β-CyD; E, with G_2-β-CyD. The dotted line represents the mean unfolding temperature of insulin without CyDs.

states. Under the present condition, the thermal unfolding of insulin was completely irreversible, since no endothermic peak was observed by rescanning the sample after it was cooled from the first run. In 30% (v/v) acetic acid solution, insulin exists mostly as the monomer[10] and gives a broad endothermic peak around 50 °C. In phosphate buffer (pH 2.0), insulin is predominantly dimeric and shows an endothermic peak over 60 °C, following an irregular exothermic pattern due to the precipitation of insulin. By contrast, the insulin hexamer and higher order oligomers in phosphate buffer (pH 6.8) showed a mean unfolding temperature (T_m) of approximately 70 °C, where large changes in the excess heat capacity may be due to the dissociation of insulin oligomers, a process which is known to be endothermic.[18] These results clearly indicate that self-association of the insulin molecules stabilized the native conformation of the peptide, and the T_m value of the peptide was a diagnostic measure for the conformational changes of the peptide.

Figure 5 shows the effects of CyDs on the excess heat capacity curves of insulin solution at pH 6.8. G_2-β-CyD and SBE4-β-CyD significantly reduced the T_m value of insulin oligomers, the former being more effective. These CyD derivatives may shift the equilibrium in favor of the unfolded insulin by dissociating the oligomers and/or binding to hydrophobic side chains exposed on the unfolded peptide.[25] Although SBE7-β-CyD promoted the insulin aggregation, it either did not affect or actually reduced slightly the T_m value of insulin. This indicates

that the conformational energy of the unfolded insulin is reduced by incorporating the exposed hydrophobic groups in the unfolding peptide into the CyD cavity, which may compensate fully for the thermal stabilization arising from the accelerated association of the peptide. On the other hand, S-β-CyD, having a limited inclusion ability, increased the T_m value of insulin by approximately 10 °C, resulting solely from the higher degree of association of the peptide.

Effects of CyDs on the ^{1}H-NMR Spectrum of Insulin In solution, an insulin monomer is in equilibrium with the dimer and hexamer.[1] Of the three insulin species, the monomer is the most likely to interact with the hydrophobic cavity of CyDs, and the primary targets for complexation would be the aromatic side chains in the peptide. In this study, further insight into the interaction mode of insulin with the CyD derivatives was gained by employing ^{1}H-NMR spectroscopy in deuterium oxide (D_2O) containing 30% (v/v) deuterated acetic acid (CD_3COOD). This solvent system weakened the self-association of insulin, enabling the monomer to be the predominant species.[10]

Figure 6 shows the effects of G_2-β-CyD (100 mM) and SBE7-β-CyD (0.5 mM) on the ^{1}H-NMR spectrum of the aromatic region of insulin (2 mM) in 30% (v/v) CD_3COOD solution at 25 °C. The concentration of SBE7-β-CyD was limited to 0.5 mM or less because of the precipitation of insulin due to the neutralization of cationic charges in the peptide by anionic sulfonate groups of the CyD. Similarly, even with lower concentrations of S-β-CyD, insulin was precipitated and the ^{1}H-NMR data could not be obtained under the acidic condition used. Table 2 summarizes the ^{1}H-NMR chemical shift displacements of assignable aromatic protons of insulin upon the addition of CyDs. As shown in Fig. 6-A, the C2 protons of the B5- and B10-histidines were well dislocated from the main aromatic envelope (6.2—7.0 ppm), and the peak height ratio of the C2 proton signal of the B10-histidine to that of the B5-histidine (B10/B5 ratio) is known to be affected by changes in the concentration of insulin in a manner that reflects the self-association of insulin.[22] Judging from the B10/B5 ratio in Fig. 6-A, insulin is confirmed to be primarily monomeric at a concentration of 2 mM.[10]

Upon the addition of G_2-β-CyD, the ^{1}H-NMR signals

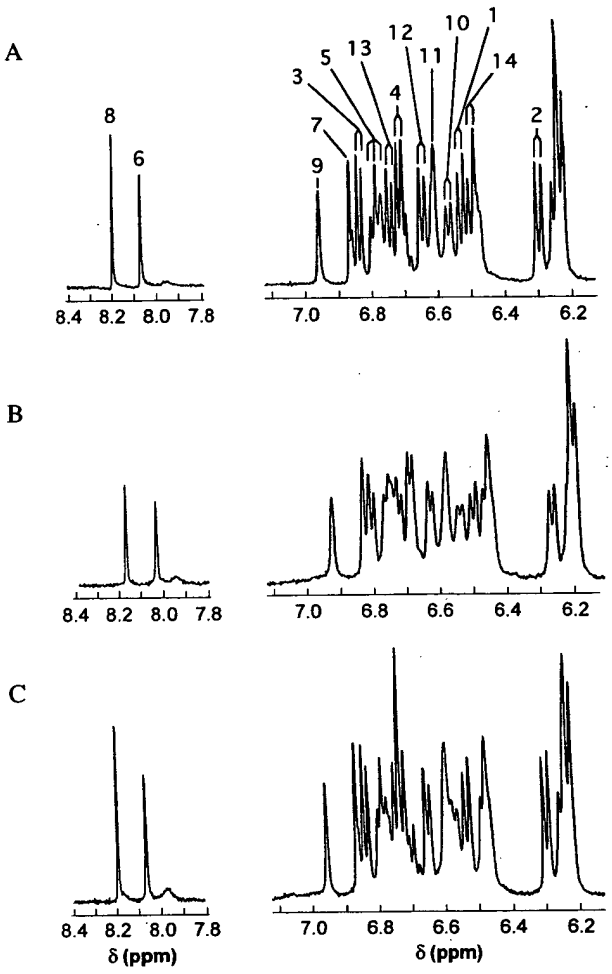


Fig. 6. Effects of G_2-β-CyD (100 mM) and SBE7-β-CyD (0.5 mM) on ^{1}H-NMR Spectrum of Aromatic Region of Insulin (2 mM) in D_2O Containing 30% (v/v) CD_3COOD at 25 °C

A, insulin alone; B, with G_2-β-CyD; C, with SBE7-β-CyD. The numbers above the peaks are the same as in Table 2.

Table 2. Effects of G_2-β-CyD (100 mM) and SBE-β-CyDs (0.5 mM) on ^{1}H-NMR Chemical Shifts of Insulin (2 mM) in 30% (v/v) Deuterated Acetic Acid at 25 °C

Number	Side chain	Position	Insulin alone (2 mM) Chemical shift (δ)	With β-CyDs, $\Delta\delta^{a)}$ (ppm)		
				With G_2-β-CyD	With SBE4-β-CyD	With SBE7-β-CyD
1	Tyrosine (A14)	C2 and 6	6.530	0.000	0.001	0.008
2	Tyrosine (A14)	C3 and 5	6.295	0.001	0.001	0.009
3	Tyrosine (A19)	C2 and 6	6.833	0.004	0.001	0.007
4	Phenylalanine (B1)	C2 and 6	6.713	0.006	0.004	0.018
5	Phenylalanine (B1)	C4	6.784	−0.001	0.000	0.007
6	Histidine (B5)	C2	8.063	−0.003	−0.001	0.008
7	Histidine (B5)	C4	6.864	−0.001	0.000	0.005
8	Histidine (B10)	C2	8.195	−0.001	0.002	0.006
9	Histidine (B10)	C4	6.955	0.000	−0.001	0.004
10	Tyrosine (B16)	C2 and 6	6.568	0.002	0.001	0.008
11	Phenylalanine (B24)	C3 and 5	6.611	−0.001	−0.006	−0.012
12	Phenylalanine (B25)	C2 and 6	6.647	0.012	0.001	0.009
13	Phenylalanine (B25)	C3 and 5	6.743	0.010	−0.001	0.003
14	Tyrosine (B26)	C2 and 6	6.498	−0.005	−0.004	−0.010

a) $\Delta\delta = \delta_{\text{with CyD}} - \delta_{\text{insulin alone}}$. Negative signs indicate upfield displacement.

of the B26-tyrosine were shifted upfield, while those of the A19-tyrosine and the B1- and B25-phenylalanines were shifted downfield. The G_2-β-CyD-induced chemical shift displacements and line broadening would be ascribable to the shielding effect due to the inclusion of the aromatic side chains within the CyD cavity[26] and/or the concomitant conformational changes in the peptide. β-CyD derivatives are reported to have relatively small binding constants of less than $200\,M^{-1}$ with free aromatic amino acids at nearly neutral pH, with the efficacy decreasing in the order of tyrosine > phenylalanine ≫ histidine, depending on their hydrophobicity[27] and on the complementary strength between host and guest molecules.[28,29] Under the acidic condition used in this study at a pH meter reading of 1.9, the imidazole moiety of the B5 and B10 histidines was completely protonated since its pK_a is *ca.* 7.0[30]; this may further destabilize such complexation.

Lovatt *et al.* have demonstrated calorimetric dilution data for insulin: CyD systems (25 °C, pH 2.5) fitted to a model assuming a sequential binding of CyDs to at least two possible sites on the insulin monomer, with corresponding binding constants of 10—$20\,M^{-1}$ and $<5\,M^{-1}$ for high and low affinity sites, respectively.[18] Based on the results in Table 2 and on the inclusion ability for the three amino acids, G_2-β-CyD may include accessible hydrophobic amino acid residues such as phenylalanine and tyrosine in the N-terminal end (B1) and C-terminal region (B25 and B26) of the B-chain, these side chains having a high motional freedom,[31] while the side chains in the α-helices (A14 and B16) are not significantly perturbed in the presence of G_2-β-CyD.

In general, the topological constraints of the peptide backbone may reduce the formation of inclusion complexes.[28] The B24-phenylalanine is known to be directed toward the hydrophobic interior of the insulin molecule and its ring rotation is considerably restricted,[32] as indicated by a remarkable line broadening of the resonances (Fig. 6-A). Therefore, no noticeable change in the ^{1}H-NMR signal of the B24-phenylalanine by the addition of G_2-β-CyD may be ascribable to the difficulty of the CyD in gaining access to the folded side chain. Also evident was the deshielding of the C2 and C6 protons of the A19-tyrosine in the presence of G_2-β-CyD. Two-dimensional NMR studies have demonstrated that the B25-phenylalanine is flexible in solution and turns transiently toward the A-chain, touching a stably folded A19-tyrosine.[14] Accordingly, a possible explanation for the deshielding may reside in the perturbation of such intramolecular interaction between the side chains, probably through the inclusion of the B25-phenylalanine within the CyD cavity. These results suggest that G_2-β-CyD includes accessible hydrophobic side chains within the CyD cavity and hence perturbs the intermolecular hydrophobic contacts between aromatic side chains across the monomer-monomer interfaces, eventually leading to the inhibition of self-association of the peptide.

As the concentration of insulin increased, the B10/B5 ratio decreased, along with a remarkable line broadening of the main aromatic resonances. Furthermore, the proton signals for the B10, B16, B24 and B26 residues were shifted upfield, while those for the A14, B1, B5 and B25 residues were shifted in the opposite direction. These spectral changes suggest the dimerization of insulin forming an antiparallel β-sheet structure between the two C-terminal strands of the B chain.[1] Similar spectral changes are expected for the insulin: SBE7-β-CyD system, because SBE7-β-CyD enhanced the self-association of insulin at neutral pH. As shown in Fig. 6 and Table 2, even with the low concentration of SBE7-β-CyD, large chemical shift changes were observed for the B24 and B26 residues (upfield-shift) and the B1 residue (downfield-shift), while the B10/B5 ratio was not much changed. SBE4-β-CyD at a concentration of 0.5 mM showed a small but essentially identical pattern of ^{1}H-NMR chemical shift changes of insulin, as compared with those observed for SBE7-β-CyD (Table 2). These spectral changes may arise from the electrostatic interaction between the positive charges in insulin and the negative charges in SBE-β-CyD rather than the inclusion complexation, and are somewhat different from those derived from the concentration-dependent dimerization of the peptide. Further NMR studies are under way to elucidate the detailed mode of interaction of insulin with the CyD derivatives.

In conclusion, the present results clearly suggest that G_2-β-CyD and anionic β-CyDs interact with insulin in a varying manner, modifying the self-association and thermal behavior of the peptide, and hence a proper use of the CyD derivatives could be effective in designing rapid or long-acting insulin preparations.

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TI ***Minimizing*** the ***aggregation*** of neutral ***insulin*** solutions

AU Quinn, R.; Andrade, J. D.

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Minimizing the Aggregation of Neutral Insulin Solutions

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Received November 16, 1981, from the *Department of Bioengineering, University of Utah, Salt Lake City, UT 84112.* Accepted for publication October 21, 1982.

Abstract □ Various solution additives affect the solubility and macroaggregation of insulin in buffered aqueous solutions at physiological pH. The solubility of insulin may be improved with the addition of small amounts of aspartic acid, glutamic acid, EDTA (ethylenediaminetetraacetic acid), lysine, Tris buffer, or bicarbonate buffer. In addition, the propensity of dissolved insulin to reaggregate and precipitate may be inhibited by such additives. Buffered physiological (pH 7.4) saline solutions containing 0.001–0.003 *M* lysine in the presence of 0.005 *M* EDTA or 0.01 *M* lysine in the absence of EDTA improve insulin solubility and are effective in minimizing aggregation. Solutions thus prepared may be suitable for application in intravenous insulin infusion devices and may be useful commercial insulin preparations.

Keyphrases □ Insulin—minimizing aggregation, neutral solutions, lysine, solubility □ Aggregation—minimization, solubility of neutral insulin solutions, lysine solubility □ Lysine—minimizing aggregation of neutral insulin solutions, solubility □ Solubility—minimizing aggregation of neutral insulin solutions, lysine

The tendency of insulin solutions to form macroaggregates is an obstacle in the development of long-term insulin delivery systems (1–5). The macroaggregation of the insulin molecule often limits prolonged infusion to a few days unless the device is regularly flushed during the test period. This problem, as well as a desire to characterize the adsorption of insulin, have led us to search for a physiological solvent or additive that will stabilize insulin solutions. Insulin solubility and prolonged prevention of macroaggregation has been achieved by addition of various agents to dilute insulin solutions (4–8).

EXPERIMENTAL

The Tris buffer contained 0.1 *M* NaCl, 0.005 *M* EDTA (ethylenediaminetetraacetic acid) (Tris-HCl 14.04 g/liter; Tris, 1.34 g/liter)[1]. The phosphate-buffered saline solution was prepared using 1.36 g of Na_2HPO_4, 0.22 g of KH_2PO_4, 0.005 *M* EDTA, and 8.5 g of NaCl/liter (0.01 *M* phosphate and 0.145 *M* NaCl). The pH of both solutions was adjusted to 7.2–7.4, as needed, by addition of 0.1 *M* HCl or 0.1 *M* NaOH. Bicarbonate buffer was prepared using 1.428 g of $NaHCO_3$ and 8.070 g of NaCl diluted to 1 liter. A mixture of 5% CO_2 and compressed air was bubbled through the solution to adjust the pH to 7.4. Amino acids and other additives were added to the buffered solutions in varying concentrations as desired.

Crystalline insulin[2] at a potency of 25.2 U/mg was used in an attempt to regulate solution additives. Many other studies have used commercially available insulin preparations which usually contain additives that influence solubility and aggregation.

Solutions of 1 ml were sealed with paraffin film in 16-ml glass tubes (16 mm × 100 mm) and continuously agitated in a shaking water bath at 100–200 cycles/min and 37°. Solution turbidity was evaluated twice daily. The degree of aggregation of the solution was assessed visually on a five-plus scale: (+) meant clear, no observable particles, and (+++++) meant large aggregates or cloudy. Initially instrumental turbidity measurements were used to assess the degree of aggregation, but because of the macroscopic nature of the aggregate, this method did not accurately reflect the amount of aggregation. "First day" results indicate apparent solubility of insulin after 2–4 hr. The "5–6 day" results indicate degree of aggregation present at that time.

RESULTS

Additives tested were aspartic acid, EDTA, glutamic acid, bicarbonate buffer, ethanol, glycerol, leucine, lysine, and Tris buffer. When increased solubility or prolonged prevention of aggregation was observed, an attempt was made to determine the minimum amount of the additive required to produce the observed result. This was done by serially diluting the additive in the buffered solution while other buffer conditions were held constant. Results are given in Table I.

Ethanol, Glycerol, and Leucine—These three compounds proved to be very unsatisfactory as additives in the concentration range tested (0.001–0.1 *M*). None of the compositions demonstrated delayed onset

Table I—Effect of Additives on Insulin Aggregation

Major Additive	Buffer[a]	pH	EDTA (0.005 *M*)	Insulin Concentration, mg/ml	Effective in Blocking Aggregation?
Lysine (0.0005–0.1 *M*)	PBS	7.4	+	6–10	Yes
Lysine (0.0005–0.1 *M*)	PBS	7.4	–	6	Slight
Lysine (0.001–0.1 *M*)	PBS	9.0	+	6	Yes
Lysine (0.001–0.1 *M*)	PBS	9.0	–	6	Slight
Aspartic Acid (0.00005–0.05 *M*)	PBS	7.4	+	6	No
Aspartic Acid (0.00005–0.05 *M*)	PBS	7.4	–	6	No
Aspartic Acid (0.00005–0.05 *M*)	PBS	3.5	+	3–6	Yes
Aspartic Acid (0.00005–0.05 *M*)	PBS	3.5	–	3–6	Yes
Glutamic Acid (0.00005–0.05 *M*)	PBS	7.4	+	6	No
Glutamic Acid (0.00005–0.05 *M*)	PBS	7.4	–	6	No
Glutamic Acid (0.00005–0.05 *M*)	PBS	3.5	+	3–6	Yes
Glutamic Acid (0.00005–0.05 *M*)	PBS	3.5	–	3–6	Yes
Leucine (0.001–0.1 *M*)	PBS	7.4	+	6	No
Glycerol (0.001–1.0 *M*)	PBS	7.4	+	6	No
Ethanol (0.001–0.1 *M*)	PBS	7.4	+	6	No
Buffer A (0.005–0.1 *M*)[a]	PBS	7.4	+	6	Yes
Buffer A (0.005–0.1 *M*)	PBS	7.4	–	6	No
Buffer A (0.005–0.1 *M*)	Tris	7.4	+	6	Yes
Sodium Bicarbonate	$NaHCO_3$	7.2–7.4	+	0.5	Yes

[a] Key: (PBS) phosphate-buffered saline; (Tris) Tris buffer in 0.1 *M* NaCl.

[1] Chemicals were obtained from Sigma Chemical Co.
[2] Obtained from Calbiochem Behring Corp., La Jolla, Calif.; lot number 003622.

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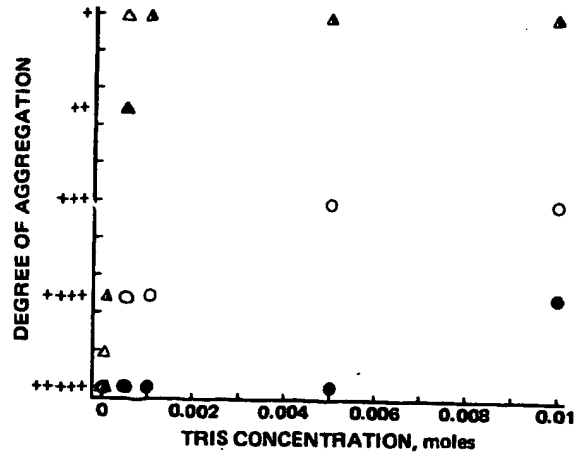

Figure 1—*Comparison of aggregation of insulin in phosphate-buffered saline as a function of Tris concentration. Solution conditions: phosphate-buffered saline, pH 7.2–7.4, temperature 37°, and insulin concentration, 6 mg/ml. Key: Degree of aggregation of solutions with 0.005 M EDTA at 1 (△) and 5 (▲) days; aggregation of solutions without EDTA at 1 (○) and 5 (●) days; (▲) point overlap.*

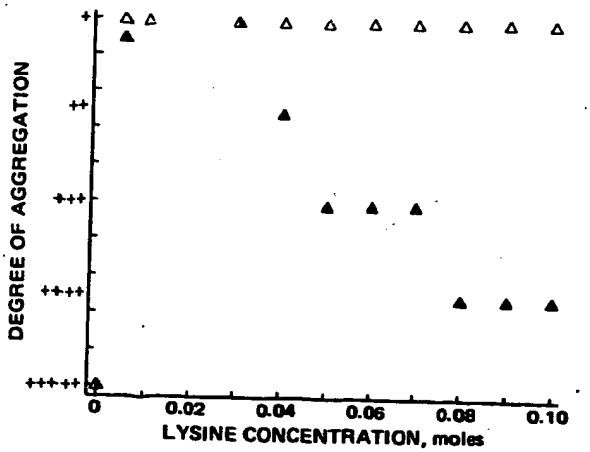

Figure 2—*Concentrations of 0.001–0.01 M lysine in 0.005 M EDTA and phosphate-buffered saline, pH 7.4. Key: degree of aggregation of solutions with 0.005 M EDTA at 1 (△) and 5 (▲) days; (▲) point overlap.*

of aggregation.

Tris Buffer—Phosphate-buffered saline solutions were prepared with and without 0.005 *M* EDTA, at various Tris concentrations (0.001–0.1 *M*). Figure 1 summarizes the aggregation of insulin as a function of Tris concentration in the presence and absence of EDTA, demonstrating that both additives are important in delaying the onset of insulin aggregation.

Lysine—Phosphate-buffered saline solutions containing lysine showed rapid dissolution of insulin, with the dissolution time decreasing as the pH was raised to 8.5–9.0. Solutions containing lysine at pH 7.2–7.4 maintained a clear, unaggregated appearance for 5–6 days. Higher lysine concentrations (0.1–0.01 *M* in 0.005 *M* EDTA) tended to aggregate more than those solutions containing lower lysine concentrations (0.01–0.001 *M*). Lysine (0.001 *M* in 0.005 *M* EDTA) is effective in minimizing aggregation (Fig. 2). However, when 0.005 *M* EDTA was eliminated from the phosphate-buffered saline solution, 0.01 *M* lysine was required to significantly minimize aggregation. Solutions of 0.01 *M* lysine and 0.005 *M* EDTA maintained at 4° without agitation remained not aggregated for periods up to 3 weeks.

Aspartic and Glutamic Acid—Earlier studies in other laboratories (5) showed that glutamic and aspartic acids were important in delaying the onset of aggregate formation. Our studies confirm the results of Bringer *et al.* (5) wherein aggregation was prevented for 6–7 days; however, serial dilution resulted in a decrease in the aggregation time. Aspartic acid proved to be more successful than glutamic acid at blocking insulin aggregation (Table I). It is important to note that due to the acidic nature of these amino acids, the pH of these solutions was 3.5 rather than 7.4. If the solutions were adjusted to pH 7.4, the aggregation was lost. This observation was also noted by Bringer *et al.* (5).

Bicarbonate—Two-milliliter solutions of sodium bicarbonate saturated with insulin were titrated to pH 6.3 with 0.1 *M* HCl, resulting in insulin precipitation. If solutions were back-titrated to pH 7.4 with 0.1 *M* NaOH the insulin remained undissolved. However, if a 5% CO_2–compressed air mixture was bubbled through the solution until pH 7.4 was reached, the insulin redissolved. A similar observation was noted by Lougheed *et al.*, where dissolution times were monitored as a function of bicarbonate concentration (8).

DISCUSSION

These results support the findings of previous researchers that agitation, additives, temperature, pH, and insulin concentration influence the solubility and macroaggregation of insulin. Recent work by Sato *et al.*, demonstrates that urea is effective in minimizing aggregation (9).

Several mechanisms for the prevention of aggregation have been proposed including the possibility of a serum substance (4) that prevents aggregation [newly published data from this group suggests that the bicarbonate concentration is the major factor in mediating insulin solubility (8)]. The chelation effect of the carboxyl groups of amino acids for zinc is believed to block aggregate formation by resulting in a more soluble form of insulin. This data is somewhat supported by the improvement of solubility and prolongation of aggregation time observed in solutions containing EDTA. As a chelating agent, EDTA may compete with insulin for zinc and, therefore, slow aggregate formation (10). Another possible mechanism for minimizing aggregation is that amino acid additives, especially lysine, may interact with the insulin molecule by hydrophobic and ionic means, thereby decreasing insulin–insulin interactions and preventing or slowing the formation of aggregates. More definitive work should be done with detailed analysis of the types of interactions and the conformation of the insulin molecule in these solutions.

Buffered physiological saline solutions containing 0.001 *M* lysine and 0.005 *M* EDTA improve insulin solubility and are effective in delaying the onset of macroaggregation. In the absence of EDTA, 0.01 *M* lysine solutions improve initial solubility and minimize the degree of aggregation. One advantage of the lysine additive is that the solutions are maintained at pH 7.4. A second advantage is that lysine is a common amino acid and is therefore not a synthetic additive.

Results summarized in this study emphasize the importance of additives in improving the solubility and stability of insulin solutions. It should be remembered that the type of insulin and additives used in various insulin preparations influence the properties discussed above, so a comparison of these results with other studies must be done with caution. The only way to accurately assess the contribution of each additive as to its solubility and aggregate-blocking properties is in a study such as this which minimizes the contributions of other solution variables or insulin additives.

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